Malik Zainul Abdin
Usha Kiran
Kamaluddin
Athar Ali *Editors*

Plant Biotechnology: Principles and Applications

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Malik Zainul Abdin • Usha Kiran
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Editors

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Preface

The group of technologies that use biological matter or processes to generate new and useful products and processes define biotechnology. The plant biotechnology is increasingly gaining importance, because it is related to many facets of our lives, particularly in connection with global warming, alternative energy initiatives, food production, and medicine. This book, entitled *Plant Biotechnology: Principles and Applications*, is devoted to topics with references at both graduate and postgraduate levels. The book traces the roots of plant biotechnology from the basic sciences to current applications in the biological and agricultural sciences, industry, and medicine. The processes and methods used to genetically engineer plants for agricultural, environmental, and industrial purposes along with bioethical and biosafety issues of the technology are vividly described in the book. It is also an ideal reference for teachers and researchers, filling the gap between fundamental and high-level approaches.

The book is comprised of 14 chapters. The first chapter is "Historical Perspective and Basic Principles of Plant Tissue Culture." It describes the use of tissue culture as an established technique for culturing and studying the physiological behavior of isolated plant organs, tissues, cells, protoplasts, and even cell organelles under precisely controlled physical and chemical environments and a source for obtaining new variants with desirable agronomic traits. It also discusses the micropropagation of the plants and its use in conservation of endangered species and afforestation programs.

The second chapter "Plant Tissue Culture: Application in Plant Improvement and Conservation" describes the use of micropropagation for ornamental and forest trees, production of pharmaceutically interesting compounds, and plant breeding for improved nutritional value of staple crop plants, including trees. It also highlights the application of plant tissue culture in providing high-quality planting material for fruits, vegetables, and ornamental plants and forest tree species throughout the year, irrespective of season and weather, thus opening new opportunities to producers, farmers, and nursery owners.

The third chapter "Plant Genetic Resources: Their Conservation and Utility for Plant Improvement" describes biodiversity as not merely a natural resource but an

embodiment of cultural diversity and the diverse knowledge of different communities across the world. The chapter reviews the genetic diversity in plant genetic resources in India, methods of its conservation, and the utilization of plant genetic resources in crop improvement programs.

The fourth chapter "Methods in Transgenic Technology" describes genetic engineering as an imperative tool for breeding of crops. The chapter reviews transgenic-enabling technologies such as *Agrobacterium*-mediated transformation, gateway vector-based technology, and generation of marker-free transgenics, gene targeting, and chromosomal engineering.

The fifth chapter "Plant Promoters: Characterization and Application in Transgenic Technology" describes the structural features of plant promoters followed by types along with examples; approaches available for promoter isolation, identification, and their functional characterization; and various transgenic crops commercialized or in pipeline in relation to the specific promoters used in their development.

The sixth chapter "Metabolic Engineering of Secondary Plant Metabolism" describes the strategies that have been developed to engineer complex metabolic pathways in plants, focusing on recent technological developments that allow the most significant bottlenecks to be overcome in metabolic engineering of secondary plant metabolism to enhance the productions of high-value secondary plant metabolites.

The seventh chapter "Plastome Engineering: Principles and Applications" summarizes the basic requirements of plastid genetic engineering and control levels of expression of chloroplast proteins from transgenes. It also discusses the current status and the potential of plastid transformation for expanding future studies.

The eighth chapter "Genetic Engineering to Improve Biotic Stress Tolerance in Plants" reviews the genes that have been used to genetically engineer resistance in plants against diverse plant pathogenic diseases.

The ninth chapter "Developing Stress-Tolerant Plants by Manipulating Components Involved in Oxidative Stress" describes recent advances in the defense system of plants during oxidative stress and also discusses the potential strategies for enhancing tolerance to oxidative stress.

The tenth chapter "Plant Adaptation in Mountain Ecosystem" discusses the physiological, morphological, and molecular bases of plant adaptation including secondary metabolism at varying altitudes in context to representative plant species in western Himalaya.

The eleventh chapter "Drought-Responsive Stress-Associated MicroRNAs" summarizes the recent molecular studies on miRNAs involved in the regulation of drought-responsive genes, with emphasis on their characterization and functions.

The twelfth chapter "Molecular Marker-Assisted Breeding of Crops" describes the molecular markers, their advantages, disadvantages, and the applications of these markers in marker-assisted selection (MAS) in crop plants to improve their agronomic traits.

The thirteenth chapter "Plant-Based Edible Vaccines: Issues and Advantages" reviews the recent progress made with respect to the expression and use of plant-derived vaccine antigens.

The fourteenth chapter "Biosafety, Bioethics, and IPR Issues in Plant Biotechnology" reviews the IPRs, biosafety, and ethical issues arising from the research in plant biotechnology and product obtained thereof.

Each chapter has been written by one or more eminent scientists in the field and then carefully edited to ensure thoroughness and consistency. The book shall be valuable for undergraduate and postgraduate students as a textbook and can also be used as a reference book for those working as plant biologists, biochemists, molecular biologists, plant breeders, and geneticists in academia and industries.

New Delhi, India Malik Zainul Abdin
New Delhi, India Usha Kiran
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List of Abbreviations and Symbols

ACT	Artemisinin-based combination therapy
ADS	Amorpha-4,11-diene synthase enzyme
BA	6-Benzyladenine
BAP	Benzene amino purine
BLAST	Basic local alignment search tool
bp	Base pair
cDNA	Complementary DNA
Cm	Centimeter
CPPU	N-(2-Chloro-4-pyridyl)-N'-phenylurea
C-TAB	Cetyl trimethyl ammonium bromide
cv./cvs	Cultivar/s
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetate
g/1	Grams per liter
gfw	Gram fresh weight
HMGR	Hydroxy methyl glutaryl coenzyme A reductase
hmgr	Hydroxy methyl glutaryl coenzyme A gene
hrs	Hours
Kb	Kilobase pairs
kDa	Kilodalton
Kn	Kinetin
MemTR	Meta-methoxy topolin
MemTTHP	Meta-methoxy topolin 9-tetrahydropyran-2-yl
mg/L	Milligram per liter
min.	Minute
mM	Millimolar
ml	Millimeter
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
NOS	Nopaline opine synthase

*npt*II	Neomycin phosphotransferase gene
°C	Degree Celsius
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	A Ribonuclease A
rpm	Rotations per minute
RT-PCR	Real-time polymerase chain reaction
sec.	Second
sp.	Species
TE	Tris-EDTA buffer
v/v	Volume/volume
var.	Variety
w/v	Weight/volume
YEM	Yeast extract mannitol
2,4-D	2,4-Dichlorophenoxy acetic acid
2-iP	2-Isopentenyl-adenine
µM	Micromolar
µl	Microliter
%	Percent

Chapter 1
Historical Perspective and Basic Principles of Plant Tissue Culture

Anwar Shahzad, Shiwali Sharma, Shahina Parveen, Taiba Saeed, Arjumend Shaheen, Rakhshanda Akhtar, Vikas Yadav, Anamica Upadhyay, and Zishan Ahmad

Abstract In 1902 Gottlieb Haberlandt proposed the idea to culture individual plant cells on artificial nutrient medium. Although he failed to culture them due to poor choice of experimental materials and inadequate nutrient supply, he made several valuable predictions about the nutrients' requirement for in vitro culture conditions, which could possibly induce cell division, proliferation and embryo induction. Tissue culture has now become a well-established technique for culturing and studying the physiological behaviour of isolated plant organs, tissues, cells, protoplasts and even cell organelles under precisely controlled physical and chemical conditions. Micropropagation is one of the most important applications of plant tissue culture. It provides numerous advantages over conventional propagation like mass production of true-to-type and disease-free plants of elite species in highly speedy manner irrespective of the season requiring smaller space and tissue source. Therefore, it provides a reliable technique for in vitro conservation of various rare, endangered and threatened germplasm. Micropropagation protocols have been standardized for commercial production of many important medicinal and horticultural crops. Somatic embryogenesis is an extremely important aspect of plant tissue culture, occurring in vitro either indirectly from callus, suspension or protoplast culture or directly from the cell(s) of an organized structure. Advantages of somatic embryogenesis over organogenesis include several practical means of micropropagation. It reduces the necessity of timely and costly manipulations of individual explants as compared to organogenesis.

Moreover, somatic embryogenesis does not require the time-consuming subculture steps. As somatic embryos are the bipolar structures, they overcome difficulties with micropropagation of difficult to root species (mainly recalcitrant tree species). In addition to micropropagation, plant tissue culture is extensively used for the production of secondary metabolites through callus, suspension and organ culture.

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1.1 History of Plant Tissue Culture

The science of plant tissue culture originally starts from the discovery of cell followed by the concept of cell theory (Schleiden 1838; Schwann 1839). Initial attempts to demonstrate ability of plant cell to regenerate into complete plantlet (totipotency) failed due to improper selection of tissue to culture, nutrient supply and culture conditions (Haberlandt 1902). Breakthrough was achieved during 1930 with the successful culturing of fragments from embryos and roots (Kotte 1922; Molliard 1921; Robbins 1922). Auxin, indole-3-acetic acid (IAA), was the first plant growth regulator (PGR) discovered by Went (1926). In 1934, first successful continuous culture of excised tomato root tips was achieved by White on sucrose and yeast extract (YE). Later, YE was replaced by vitamin B, namely, pyridoxine (B_6) and thiamine (B_1). The same year (1934) witnessed one of the main events in the history of tissue culture, the callus induction from woody cambial explants of oak (Gautheret 1934). Later in 1939, Gautheret, White and Nobécourt independently worked for the formation of continuous callus cultures in carrot and tobacco. By adding adenine and high concentrations of phosphate, continued induction of cell division and bud formation were achieved (Skoog and Tsui 1951). Kinetin (Kn), a derivative of adenine (6-furfuryl amino purine), was isolated in 1955 (Miller et al. 1955). Miller et al. (1955), Skoog and Miller (1957) also proposed the concept of hormonal control for organ formation and suggested that high concentration of auxin is required for root induction, while for bud formation, comparatively high concentration of natural cytokinin, i.e. kinetin, is required.

The most significant success in plant tissue culture was the formulation of a defined culture medium (Murashige and Skoog 1962). Murashige and Skoog used 25 times higher concentration of salts than Knop's solution. Nowadays, Murashige and Skoog (MS) medium has been proved as the most effective culture medium for most of the plant species.

1.2 Steps Involved in Plant Tissue Culture

1.2.1 Establishment of Culture

Explants (i.e. excised plant parts), viz. nodes, shoot tips, leaves, internodes, flower buds, petioles, leaflets, etc., collected from in vivo grown sources are usually contaminated with microorganisms of different types and constitution in the form of surface contaminants. Besides these, endophytic bacteria and fungi can express themselves in culture even after years.

Washing of explants with common sterilizing agents like sodium or calcium hypochlorite (5–10 %), ethyl alcohol (50–95 %) and mercuric chloride (0.01–0.1 %) in the appropriate solution for 1–30 min, followed by several rinses in sterilized water, is suggested to exclude the surface contaminants. It should be followed by

Calcium Hypochlorite (9-10%) (5-30 min)
Sodium Hypochlorite (2%) (5-30 min)
Antibiotic (5-50 mg/l) (30-60 min)
Surface sterilizing agents
Hydrogen peroxide (10-12%) (5-15 min)
Mercuric chloride (0.01-0.1%) (2-10 min)
Bromine water (1-2%) (2-10 min)
Silver nitrate (1%) (5-30 min)

Fig. 1.1 Agents used for surface sterilization

rigorous screening of the stock cultures for bacterial contamination (Murashige and Skoog 1962; Rout et al. 2000). The most common surface sterilizing agents along with range of exposure time are given in Fig. 1.1.

Axenic cultures are developed, mostly in tree species, in order to combat the contaminants. For this, first explants are taken from in vivo grown mature trees and, thereafter, cultured in vitro on MS basal medium to raise single or multiple axillary shoots which in turn are used as explant source. Such explants have advantage over direct explants, as there are lesser chances of infection and they are true to type.

Another technique to check out contamination is to use seedling-derived explants. A large number of plants have been propagated through this technique where seeds are either collected or purchased, from a reliable source, are surface decontaminated following a regular washing protocol and are thereafter transferred to germination media. After germination, healthy seedlings are sacrificed, and different types of explants are used for further propagation studies. Reliable protocol has been developed for micropropagation of *Gymnema sylvestre* through seedling-derived explants (Komalavalli and Rao 2000). Aseptic seedling-derived young root segments were used for in vitro propagation of *Clitoria ternatea* (Shahzad et al. 2007), while seedling-derived cotyledonary explant was used for micropropagation in *Cassia sophera* (Parveen et al. 2010). Seedling-derived nodal segment was used for somatic embryogenesis in *Hygrophila spinosa* (Varshney et al. 2009). The only problem associated with seedling-derived explants is variation (Larkin and Scowcroft 1981). Different procedures or techniques are carried out by various workers to eradicate the above-mentioned problems, while the most common protocol followed is summarized in Fig. 1.2.

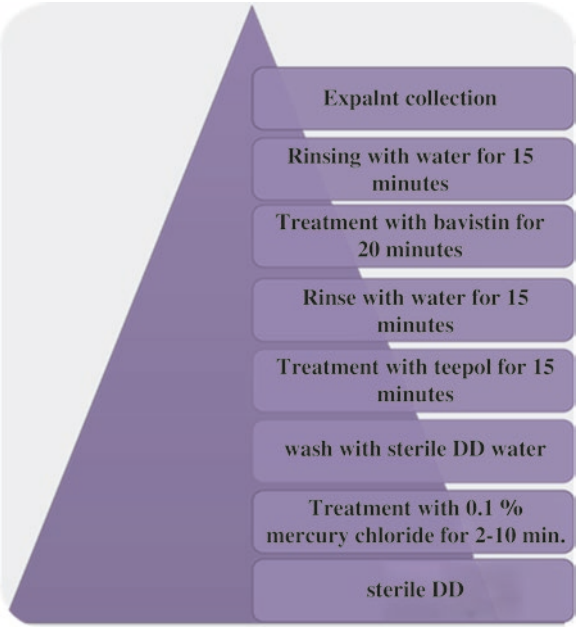
Expalnt collection
Rinsing with water for 15 minutes
Treatment with bavistin for 20 minutes
Rinse with water for 15 minutes
Treatment with teepol for 15 minutes
wash with sterile DD water
Treatment with 0.1 % mercury chloride for 2-10 min.
sterile DD

Fig. 1.2 Schematic representation of protocol for surface sterilization

1.2.2 Selection of Media

A nutrient medium consists of all the essential major and minor plant nutrient elements, vitamins, plant growth regulators and a carbohydrate as carbon source with other organic substances as optional additives. Components of media can be classified into five groups:

1. Inorganic nutrients
   (a) Macronutrients
   (b) Micronutrients
2. Organic nutrients
3. Carbon source
4. Solidifying agent
5. Growth regulators

Sucrose is generally used at a concentration of 3 % as a carbon source in plant tissue culture medium. Agar is most commonly used for preparing semisolid or solid culture media, but other gelling agents are occasionally used including gelatin, agarose, alginate and gelrite.

There are several culture media proposed from time to time for various purposes. More than 50 different devised media formulations have been used for in vitro culture of tissues from various plant species (Heller 1953; Murashige and Skoog 1962; Eriksson 1965; Nitsch and Nitsch 1969; Nagata and Takebe 1971; Schenk and

Hildebrandt 1972; Chu 1978; Lloyd and McCown 1980), but MS medium is most commonly used, often with relatively minor changes (Rout et al. 2000).

1.2.3 Selection of Plant Growth Regulators (PGRs)

Hormones are organic compounds naturally synthesized in higher plants which influence growth and development. There are two main classes of growth regulators used in tissue culture, auxin and cytokinins. The hormonal content of a cultural medium is crucial to any sustained growth of the cultures (Bhojwani and Razdan 1996). The growth regulators are required in very minute quantities (μmol l^{-1}). There are many synthetic substances having growth regulatory activity, with differences in activity and species specificity. It often requires testing of various types, concentrations and mixtures of growth substances during the development of a tissue culture protocol for a new plant species. The most important are auxins, abscisic acid, cytokinins, ethylene and gibberellins.

1.2.4 Incubation Conditions

Rout et al. (2000) stated that light, temperature and relative humidity are important parameters in culture incubation. Photosynthetic activity is not very important during initial phases of in vitro culture, but at later stages, the culture materials are induced to become autotrophic to a certain degree. Light is essential for morphogenetic processes like shoot and root initiations and somatic embryogenesis. Both quality and intensity of light as well as photoperiod are very critical to the success of certain culture experiments (Murashige 1977). An exposure to light for 12–16 h per day under 35–112 μmol m^{-2} s^{-1} provided by cool, white fluorescent lamps is usually preferred. Murashige (1977) stated that blue light promotes shoot formation, whereas rooting in many species is induced by red light. The temperature is usually maintained at 25 °C in the culture room with certain variations such as higher temperature which is usually required by tropical species (i.e. 27–30 °C; Tisserat 1981).

1.3 Micropropagation

Micropropagation is one of the most useful aspects of plant tissue culture technique. It has found widest practical application. The process of micropropagation involves the following four distinct stages (Murashige 1974). The first stage is culture initiation which depends on explant type or the donor plant at the time of excision. Explants from actively growing shoots are generally used for mass scale

multiplication. The second stage is shoot multiplication which is crucial and achieved by using plant growth regulators (PGRs) generally, auxins and cytokinins. In the third stage, elongated shoots are subsequently rooted either ex vitro or in vitro. The fourth stage is acclimatization of in vitro grown plants, which is an important step in micropropagation.

1.3.1 Organogenesis

Organogenesis, in terms of plant tissue culture, can be defined as the 'genesis' or formation of organs from unusual parts (i.e. adventitious development of organs). The adventitious origin may be attributed to either direct differentiation of cells and tissues (explants) to form an organ or via cells undergoing cycles of dedifferentiation (caulogenesis) and redifferentiation. In normal in vitro conditions and under the influence of various factors, organogenesis is a two-step process where shoots develop first and roots next, giving rise to a complete plantlet.

The tenets of organogenesis are based upon the fundamentals of in vitro cell culture which was initiated as early as 1898 by a German botanist, Gottlieb Haberlandt (1902). He isolated and cultured fully differentiated and mature cells of leaves and petiole on Knop's salt solution (1865) containing glucose and peptone, maintained under aseptic condition. His attempts were limited to the growth of cells in size and change in shape, but no growth in number of cells could be observed as none of the cells showed division. Much later, Skoog (1944) and Skoog and Tsui (1951) demonstrated callus growth and bud initiation in tobacco pith tissues in the presence of adenine and IAA. Later, Jablonski and Skoog (1954) confirmed cell division only when vascular tissues were present and pith cells alone were inefficient in inducing cell division. The technique of tissue culture relies upon certain internal and external factors which determine organogenesis. The internal factor mainly includes genotype and endogenous levels of growth regulators. Among the external factors, explant type, season of explant harvesting and culture room conditions (temperature, light, humidity, etc.) play pivotal role in overall development of cultured plants.

1.3.1.1 Effect of Plant Growth Regulators (PGRs)

PGRs play important role in cellular programming in manipulation of cell tissues in vitro (Moyo et al. 2011) through which morphogenic changes (viz. organogenesis, rhizogenesis, embryogenesis, etc.) take place. During micropropagation, the incorporation of exogenous cytokinin in the medium enhances shoot formation, and, for developing a standard plant tissue culture (PTC) protocol, the selection of cytokinin is of critical importance (Sharma et al. 2010, 2014; Sharma and Shahzad 2013; Parveen and Shahzad 2014a).

The effect of different PGRs has early been studied by Sahai and Shahzad (2013) in *Coleus forskohlii*, where BA (5 μM) in MS medium produced 13.80 ± 1.24 axillary shoots and 18.80 ± 1.59 direct adventitious shoots per explant. Rani and Rana (2010) studied the effects of Kn, BA and GA_3 in *Tylophora indica*. The shoot development showed dependency on synergistic effect of BA (2 mg/l) + GA_3 (0.2 mg/l) giving 4.86 ± 1.76 shoots/explant. Parveen et al. (2010) reported maximum shoot regeneration frequency with maximum number of shoots per explant (12.20 ± 0.73) and shoot length (6.40 ± 0.07 cm) on MS + BA (1.0 μM) + NAA (0.5 μM) through cotyledonary node explant, excised from 14-day-old aseptic seedlings. Similarly, in *Heliotropium kotschyi*, a synergistic effect of BA (8.88 μM) + IAA (5.71 μM) showed formation of 10.66 shoots per explant (Sadeq et al. 2014). Likewise, Ragavendran et al. (2014) reported 7.7 ± 1.1 shoots/explant in *Eclipta alba* in a combination of BA (0.5 mg/l) + Kn (0.3 mg/l) + GA_3 (1.5 mg/l) augmented in B_5 medium with 100 % regeneration frequency (Table 1.1).

1.3.1.2 Effect of Explant Type

The effect of explants on micropropagation has also been studied in various plant species such as *Gerbera jamesonii* (Tyagi and Kothari 2004), *Vitis vinifera* (Jaskani et al. 2008), *Citrus jambhiri* (Vijaya et al. 2010), *Stevia rebaudiana* (Sharma and Shahzad 2011) and *Tectona grandis* (Kozgar and Shahzad 2012). Explant-dependent micropropagation protocol has also been cited by many in different medicinal plants. Golec and Makowczynska (2008) studied the effects of seedling-derived explants of *Plantago camtschatica* on multiple shoot formation. Out of root, hypocotyl, cotyledon and leaf explants, they obtained best multiplication results from root explants giving out 12.7 ± 10 buds and shoots at 9.1 μM zeatin in combination with 0.6 μM IAA. In *Tectona grandis*, shoot tip proved to be the best for propagation as compared to nodal segments and cotyledonary nodes (Kozgar and Shahzad 2012). Micropropagation studies on different explants of *Bacopa monnieri* (Kumari et al. 2014) showed development of 18.8 ± 0.40 shoots per nodal explants as compared to shoot tip explants, which developed 14.6 ± 0.26 shoots per explant in MS + BA (0.5 mg/l) + Kn (0.5 mg/l) + IBA (0.25 mg/l) augmented medium. Jesmin et al. (2013) reported encouraging results from nodal explants (12.2 ± 0.32 shoots/culture) as compared to ST explants on the same medium, i.e. MS + BA (1 mg/l) showing 90 % regeneration rate in a period of only 10–11 days (Table 1.2).

1.3.1.3 Effect of Seasonal Variation

Bhatt and Dhar (2004) found that shoot collection season reduces percent browning and induces bud break in *Myrica esculenta.* The season of inoculation of explant as reported by Mannan et al. (2006) in *Artocarpus heterophyllus* describes survivability of shoot buds and their proliferation. A well-defined regeneration protocol showing seasonal variation has been discussed by Malik and Wadhwani (2009) for *Tridax*

Table 1.1 Effect of plant growth regulators

Plant	PGR	Explant	Medium	Observation	References
Coleonema album	BA,Kn, mT, MemTR, MemTTHP, TDZ	ST, young leaves, petiole of young leaves, stem cuttings	MS	Among various cytokinins tested mT (5 µM) supplemented in MS medium produced 14.5 shoots/ST explant, surpassing the other PGRs tested. The effects of KIN didn't influence organogenesis much when compared to the control	Fajinmi et al. (2014)
Dendrobium chrysanthum	BA, TDZ, 2,4-D	Axenic nodal segments	MS	Among all the concentrations and combinations of PGRs used MS supplied with TDZ, (5 µM) + BAP (5 µM) proved to be most responsive in terms of % response (100 %) and maximum number of shoots/ explant (14.33 ± 0.14)	Hajong et al. (2013)
Ocimum basilicum	BA, 2-iP	Nodal segments	MS	MS + BA (10 µM) proved best among different concentrations of BA and 2-iP forming 5.7 ± 0.35 shoots/explant. This no. further enhanced to 13.4 ± 1.80 with the addition of glutamine (30.0 mg/L)	Shahzad et al. (2012)

(continued)

Table 1.1 (continued)

Plant	PGR	Explant	Medium	Observation	References
Cassia siamea	BA, Kn, TDZ	CN	MS	Among different PGRs used, plant responded best at BA (1.0 μM) with 80 % regeneration rate giving 8.20 ± 0.66 shoots/ explant. A combined effect of optimal concentration of BA with NAA (0.5 μM) enhanced multiplication further giving 12.20 ± 0.73 shoot/explant with 90 % regeneration frequency	Perveen et al. (2010)
Carlina acaulis	BA, Kn, Zea	ST, Hypocotyl	MS	Morphogenesis was best studied from ST explant cultured on MS + BA (4.4 μM) obtaining 7.9 ± 0.4 shoots/explant, but 100 % response was achieved on MS + BA (13.3 μM). Moreover with subculture passage no. of shoots reduced to 5.6 ± 0.4	Trejgell et al. (2009)

(continued)

Table 1.1 (continued)

Plant	PGR	Explant	Medium	Observation	References
Centaurium erythraea	BA, CPPU, 2-iP, Kn, TDZ, Zea	In vitro raised normal and hairy roots	½MS	Urea-derived PGRs like TDZ and CPPU were more effective than adenine-based PGRs in evoking morphogenesis between normal and hairy root explants. Normal roots at 3.0 μM CPPU were more effective in morphogenesis giving 25.61 ± 0.53 number of shoots	Subotic et al. (2008)

BA 6-benzyladenine, *Kn* kinetin, *CPPU* N-(2-chloro-4-pyridyl)-N′-phenylurea, *2-iP* 2-isopentenyl]-adenine, *TDZ* thidiazuron, *2,4-D* 2,4-dichlorophenoxyacetic acid, *mT* meta-topolin, *MemTR* meta-methoxy topolin, *MemTTHP* meta-methoxy topolin 9-tetrahydropyran-2-yl

procumbens. The protocol describes highest bud break and multiple shoot formation between July and September on MS + BA (1 mg/l), whereas explants inoculated during December were least responsive. Verma et al. (2011) also studied the seasonal effect on shoot proliferation through nodal segment of *Stevia rebaudiana*. Nodal segments cultured during June to August on MS + BA (0.5 mg/l) + Kn (0.5 mg/l) exhibited maximum bud break (80.5 %) and shoot multiplication (17.5 shoots/explant). While, in *Vitex negundo*, the nodes inoculated during March–May showed maximum bud break (95 %) with 7.29 ± 0.28 shoots/explant in MS medium fortified with 1 mg/l BA, but the activity declined to 26 % with only 2.20 ± 0.21 shoots/explant during September–November (Steephen et al. 2010). Seasonal effect of explant in *Glycyrrhiza glabra* has also been discussed by Yadav and Singh (2012). According to their study, nodal segments planted during May–August were more responsive with 86.6 % bud break and 3.0 ± 0.8 shoots/explant as compared to other months (Table 1.3).

1.3.1.4 Effect of Genotype

The effect of genotype has been an important aspect for plant tissue culture (PTC) mainly because an elite germplasm is sought for this purpose. A study was conducted on *Melissa officinalis* genotypes taken from different places by Mohebalipour et al. (2012). A maximum of 4.97 ± 0.20 shoots were obtained in Iranian landrace Hamadan 2 genotype, but the genotype Fars showed more shoot elongation, whereas

Table 1.2 Effect of explant type on regeneration

Plant	Explant type	Medium	Observation	References
Dianthus caryophyllus	ST, NS	MS	Highest number of shoots (4.30 shoots/explant) were achieved from nodal segments on MS + BA (2 mg/l)	Al-Mizory et al. (2014)
Curcuma caesia	Leaf, root, rhizome sections, mature bud of rhizome, sprouted bud of rhizome	MS	Sprouted buds of rhizome showed best response in a combination medium containing 4 mg/l BA and 100 mg/l adenine sulphate giving 3.8 ± 0.32 shoots/explant	Behar et al. (2014)
Bauhinia variegata	Cotyledons, hypocotyl, leaves	MS (liquid and solid media)	Direct organogenesis was best observed in liquid media supplemented with 2-iP (2 mg. dm-3) from cotyledons showing emergence of 212.2 ± 26.6 mean number of shoot buds	Banerjee (2013)
Saintpaulia ionantha	Leaf disc and petiole	MS	Leaf disc in the presence of MS + BA (0.5 mg/l) + IBA (0.5 mg/l) gave highest no. of shoot buds (80 shoots/explant)	Ghasemi et al. (2012)
Prunus microcarpa subsp. tortusa	Cotyledons, hypocotyl, root of seedling	Nasand Read mediun (2004)	Cotyledon explant exhibited maximum regeneration rate	Nas et al. (2010)
Spilanthes mauritiana DC.	ST, leaf explants	MS	A combination of BA and IAA was more efficient in inducing 18.8 ± 0.3 shoots per ST without undergoing any callus phase during the culture	Sharma et al. (2009)
Cinnamomum tamala	Petiole, apical shoot, shoot with internode, leaf	WPM	Indirect organogenesis was best achieved in petiole explant forming 4 shoots/explant in a combination of BA (2.5 μM) and IBA (5 μM)	Sharma and Nautiyal (2009)

MS Murashige and Skoog medium, *WPM* woody plant medium

genotypes Karaj and Qazvin 2 produced highest callus. Xing et al. (2010) used four genotypes of *Rosa rugosa* for regeneration studies. Genotype Purple Branch among Tang Red, Puce Dragon and Tang White was best in achieving maximum number of shoots (4.87 ± 0.51) on MS medium augmented with BA (2.2 μM) + NAA (0.054 μM) + GA_3 (0.4 μM) with glucose as the carbon source (Table 1.4).

Table 1.3 Effect of seasonal variation

Plant	Harvesting season	Medium	Observation	References
Pithecellobium dulce	Jan–March April–June July–Sept Oct–Dec	MS	Explants harvested during Oct–Dec were more responsive in giving max bud break and showed less pathogen contamination	Goyal et al. (2012)
Celastrus paniculatus	Dec–march April–July Aug–Nov	MS	90 % bud break was observed in explants taken during April–July which declined to 70 % during Aug–Nov	Yadav et al. (2011)
Tylophora indica	Dec–Feb March–May June–Aug Sept–Nov	MS	During Sep–Nov highest % bud break (95.74 ± 3.19) was observed giving 4.50 ± 0.20 no. of shoots/explant. In this case winter season (Dec–Feb) was least responsive	Rani and Rana (2010)
Lilium ledebourii	Spring Summer Winter	MS	Highest no. of bulbets/explant were observed during summer season but for the other parameters, viz. rooting, post-acclimatization survival, winter harvesting was suitable	Azadi and Khosh-Khui (2007)
Myrica esculenta	Jan–Dec	WPM	Winter season (Nov–Dec) marked maximum bud breaks and explant establishment. During spring explants died due to phenolics released from growing shoots	Bhatt and Dhar (2004)

MS Murashige and Skoog medium, *WPM* woody plant medium

1.3.1.5 Effect of Culture Room Conditions

The culture requires incubation under controlled condition which includes optimum temperature range, humidity, light quality as well as intensity and duration of photoperiod. An account of all the factors influencing culture condition has been described in Table 1.5.

1.3.2 Somatic Embryogenesis

Somatic embryogenesis (SE) is an extremely important aspect of induced regeneration, occurring in vitro, either indirectly from callus, suspension or protoplast culture or directly from the cell(s) of an organized structure such as leaf, cotyledon, stem segment or zygotic embryo. It is a complex developmental programme by which haploid or diploid competent somatic cells undergo differentiation into complete plants through various characteristic embryological stages without the

Table 1.4 Effect of genotype

Plant	Genotype	Medium	Observation	References
Arbutus unedo	AL2, AL3, AL4, AL6, AL7, IM1, IM2, IM4, IM6 AND JF3	FS basal medium (1974)	Genotype AL7 showed best morphogenic response among the other tested genotypes forming 1.90 ± 0.73 number of shoots per test tube	Gomes et al. (2010)
Buddleia cultivars	Black Knight, Royal Red, White Ball, Nanhoensis, *B. Lochinch*, Pink Delight, White Profusion, Empire Blue, Ile de France and Border Beauty	MS medium	*Buddleia* cultivars showed genotype-independent regeneration. The bisected internodes in four cultivars, viz. *Lochinch*, Border Beauty, Pink Delight and Ile de France, were more responsive in terms of number of adventitious shoot formation	Phelan et al. (2009)
Allium cepa	B-780 Hisar-2 N-2-4-1	MS medium	Among different genotypes B-780 was significantly superior in all explants studied (ST, RT seed) in inducing callus and multiple shoot formation	Khar et al. (2005)
Morus alba	Chinese white Kokuso- 27 Ichinose	MS basal medium (fortified with 0.1 mg/l TIBA)	Kokuso-27, among the three genotypes studied, was best in forming regenerative calli (90 %) and number of shoots/ callus (11.4)	Bhau and Wakhlu (2001)
Dianthus caryophyllus	Coral Jaguar Salome Sarinah	MS medium containing B_5 vitamins	Salome and Jaguar cultivars were intensively caulogenic but developed roots only. Coral and Sarinah genotypes were low caulogenic but evidenced intensive organogenic capacity developing both roots and shoots	Kallak et al. (1997)

MS Murashige and Skoog medium, *WPM* Woody plant medium

Table 1.5 Effect of culture room conditions

Plant	Factor (Light)	Medium	Explant	Observation	References
Lysionotus pauciflorus	WL, BL, OL, RL	MS with varied composition of nitrogen	Leaf	RL proved to be superior with 30.4 ± 7.5 shoots/explant and showing 100 % regeneration rate	Lu et al. (2013)
Alternanthera brasiliana	WL, RL, GL,BL	MS	Axenic nodes of germinated plantlet	BL was significant in terms of largest no. of leaf/ explant. RL resulted in formation of lower parameters	Macedo et al. (2011)
Cattleya hybrid	WL, BL, RL, FRL	MS	Shoots regenerated from protocorm-like bodies	Enhanced adventitious bud formation in RL and BL. RL promoted elongation of shoots and BL promoted rhizogenesis and elongation of aerial roots	Cybularz-Urban et al. (2007)
Alternanthera brasiliana	WL + UV-A	MS	Nodal segments	Regeneration frequency enhanced to 96 % with 100 % rooting and showed comparatively lesser value of chl a /chl b ratio	Silva et al. (2005)
	Temperature				
Mentha sp.	20 °C and 25 °C	MS	Apical and nodal explants	Nodal explants at 25 °C exhibited maximum no. of leaves	Islam et al. (2005)

intervention of a sexual fusion. Thus, the various developmental stages of somatic embryos correspond to that of zygotic embryos (Dodeman et al. 1997). Advantages of somatic embryogenesis over organogenesis include several practical means of propagation. The time-consuming subculture steps and in vitro root induction in recalcitrant plant species during organogenesis are not required during somatic embryogenesis (Thangjam and Maibam 2006). Somatic embryoids, being bipolar in organization, required a single step to get differentiated into an integrated root-shoot axis unlike the development of monopolar structures, either root or shoot through organogenesis. The origin and development of adventitious embryoids in culture was first reported by Steward et al. (1958) and Reinert (1959) in carrot cell suspension cultures. Carrot served as a model system for the detailed study of structural and developmental patterns of somatic embryogenesis, since most of the early work on somatic embryogenesis was concentrated on this plant (Wetherell and Halperin 1963; Kato 1968; Homes 1968). Since then the somatic embryogenesis has been successfully reported in many plants (Gharyal and Maheshwari 1981; Schuller et al. 1989; Martin 2004; Nowak et al. 2012) including many medicinally important plants (Murthy and Saxena 1998; Jayanthi and Mandal 2001; Kumar et al. 2002; Paramageetham et al. 2004; Ma et al. 2011). Secondary embryogenesis, i.e. phenomenon of induction of new somatic embryos in a cyclic manner from the preexisting one, is of common occurrence in many plant species. Secondary embryogenesis ensures high multiplication rate with greater uniformity of the emblings and is also independent on the explant availability (Shi et al. 2010). Also embryogenicity of an established culture could be maintained for long durations, i.e. up to many years through the process of cyclic or recurrent embryogenesis (Uzelac et al. 2007; Konan et al. 2010; Shi et al. 2010; Sahai et al. 2010; Saeed and Shahzad 2015). The responsive cells (also called as embryogenic cells) have the ability to activate embryo-responsive genes, thus leading to the initiation of the embryogenic pathway (Nomura and Komamine 1995; Quiroz-Figueroa et al. 2002). The explant changes its established gene expression programme to embryogenic gene expression as soon as the embryo responsive genes become activated (Quiroz-Figueroa et al. 2006). The key step in embryogenic induction is to determine specific factors that act as signalling molecules to change the somatic cells expression pattern towards embryogenic pathways. Internal and/or external cellular levels of plant growth regulators (PGRs), various stress factors such as osmotic shock, water stress, heavy metal ions, alterations of culture medium, pH, heat or cool shock treatments, hypoxia, antibiotics, ultraviolet radiation and mechanical or chemical treatments as well as reduced nitrogen are important inductive factors in generating signal transduction cascade leading to a series of cell division which may either give rise to unorganized embryogenic callus or polarized growth resulting into direct or indirect embryogenesis, respectively (Dudits et al. 1991; de Jong et al. 1993; Trigiano et al. 1992). Williams and Maheswaran (1986) suggested that the two pathways, direct and indirect somatic embryogenesis, proceed from different types of cells. Pre-embryogenic determined cells (PEDCs), which were already determined for embryogenic development prior to explanting, required only minimal reprograming of tissues for the expression of direct embryogenesis, while indirect embryogenesis proceeds from induced embryogenically determined cells (IEDCs)

that require major reprogramming to get proliferated calli with embryogenic ability before embryo formation. Another point of discussion is a single- or multiple-cell origin of somatic embryoids. Induction of somatic embryo from a superficial cell possibly indicates its unicellular origin (Haccius 1978) or from subepidermal cells, representing a multicellular origin (Tisserat et al. 1978). The various events occurring during somatic embryogenesis have been schematically represented in Fig. 1.3.

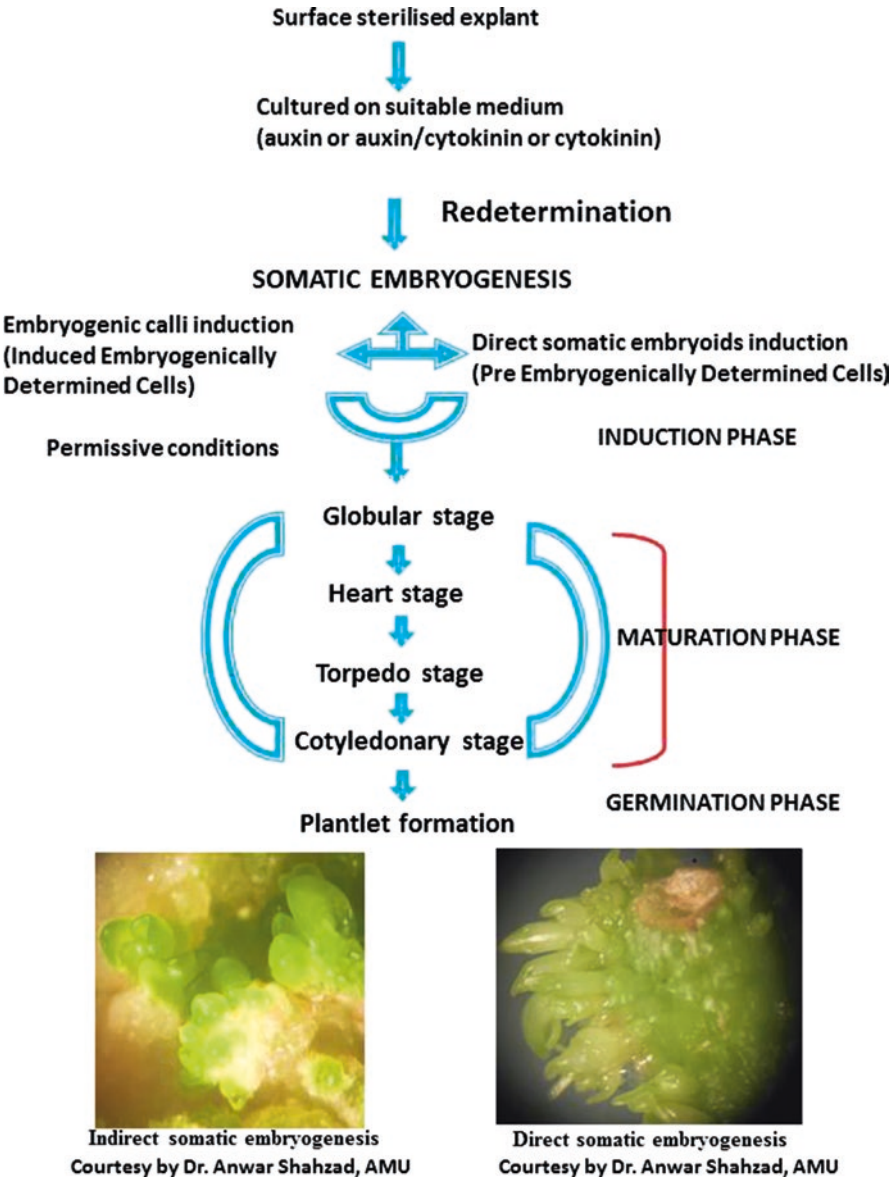

Fig. 1.3 Agents used for surface sterilization

1.3.2.1 Effect of Media

The different types of media (MS, WPM, B5, N6, SH, LS) too have a significant impact on somatic embryogenesis; MS is the most commonly used nutritional medium for the induction of somatic embryogenesis (Martin 2003; Husain et al. 2010; Silja et al. 2014). Differences in the concentrations or combinations of nutrients have a very influential role in the optimal expression of embryogenic potential. Among all the inorganic nutrients, nitrogen is a major nutrient component that influences in vitro morphogenesis of the species or the plant organ considered (Samson et al. 2006) (Table 1.6).

1.3.2.2 Effect of PGRs

PGRs are the key players in the induction of somatic embryogenesis. Auxin is considered to be the most important inducer of somatic embryogenesis in vitro (FehÈr 2008). It has been reported that 2,4-dichlorophenoxyacetic acid (2,4-D) is the

Table 1.6 Effect of media on SE of some selected medicinal plants

Plant	Explant used	Media used	Response	References
Paris polyphylla			*% SE*	
	Immature zygotic embryos	B5; ½ B5	20.7; 29.6	Raomai et al. (2014)
		MS; ½ MS	17.0; 32.6	
		N6; ½ N6	18.5; 26.6	
		SH; ½ SH	24.4; 28.9	
Murayya koengi			*% Callus induction*	
	Zygotic embryos	MS + 2.675 µM NAA + 4.44 µM BA	90 (embryogenic callus)	Paul et al. (2011)
		WPM + 2.675 µM NAA + 4.44 µM BA	63.33 (non-embryogenic callus)	
Eucalyptus globulus			*% SE*	
	Mature zygotic embryos	MS; ½ MS	30; 20	Pinto et al. (2008)
		B5	20	
		DKW	8	
		WPM	4	
		JADS	12	
Sesbania sesban			*% SE*	
	Cotyledonary explants	LS	10	Shahana and Gupta (2002)
		MT	11	
		N	9	
		MS	2	
		B5	0	
		N6	0	
		SH	0	
		W	0	

classic auxin for the induction of somatic embryos (SEs) in many species (Pasternak et al. 2002). Higher contents of endogenous auxins in embryogenic cultures than their non-embryogenic counterparts have been reported in *Medicago falcata* (Ivanova et al. 1994) and wheat (Jimenez and Bangerth 2001). Further development of the somatic embryos generally occurs through the reduction or removal of 2,4-D from the culture medium. Cytokinins are also the key determinants of embryogenic response in several plant species. BA was found suitable for SE in *Hygrophila spinosa* (Varshney et al. 2009), *Sapindus mukorossi* (Singh et al. 2015), *Albizia lebbeck* (Saeed and Shahzad 2015). Thidiazuron (TDZ), a phenylurea-derived cytokinin, has proved its potential in high frequency direct induction and development of somatic embryoids even from the mature tissues (Panaia et al. 2004; Zhang et al. 2005). The effect of GAs on SE is highly variable from one to another species or tissues, for example, GA inhibited SE in carrot (Tokuji and Kuriyama 2003), whereas it stimulated embryogenesis in petiole-derived tissue cultures of *Medicago sativa* L. (Ruduś et al. 2002). ABA was found to induce somatic embryos directly from the epidermal cells of seedlings in carrot, and the concentration of this hormone determines the number of induced embryos (Nishiwaki et al. 2000). The positive role of ABA includes normal development of plantlets from somatic embryoids, as it inhibits the precocious germination and stimulates their maturation (Kuklin et al. 1994) (Table 1.7).

1.3.2.3 Effect of Explant Types

Age, developmental stage and the physiological state of the donor plants play vital role in the induction of somatic embryos in cultured tissues. Almost any part of the plant can be used as explant to initiate embryogenic cultures such as in carrot, which is very responsive plant (Jiménez et al. 2005), whereas in other recalcitrant plants such as in cereals and conifers, very specific, usually juvenile explants are responsive for the induction of somatic embryogenesis (Bhaskaran and Smith 1990; Stasolla et al. 2002). Various tissue explants such as immature and mature zygotic embryos (Raomai et al. 2014; Rai and McComb 2002), cotyledons (Kumar et al. 2002; Parveen and Shahzad 2014b), hypocotyl (Choi et al. 1999; Kumar et al. 2002), leaf (Jayanthi and Mandal 2001; Sahai et al. 2010), petiole (Choffe et al. 2000), root (Franklin and Dias 2006), shoot (Dhandapani et al. 2008), nodal segments (Devendra et al. 2011), internode (Martin and Pradeep 2003), etc. have been exploited so far for the induction of somatic embryogenesis. Hypocotyl explants of *Catharanthus roseus* produce embryogenic callus on 1.0 mg/L 2,4-D-supplemented MS medium, while the calli induced from root, leaf and stem explants on the same medium proved to be non-embryogenic (Dipti and Mujib 2014). In another study on the same plant, direct somatic embryogenesis with maximum embryoid induction (48.7 %) was observed from mature embryos on 7.5 μM TDZ-augmented MS medium, while indirect somatic embryogenesis was induced from petiole, shoot tip as well as stem node on the same medium. Hypocotyl and cotyledon explants did not produce somatic embryoids at all. Akula et al. (2003) reported direct

Table 1.7 Effect of PGRs on SE of some selected medicinal plants

Plant	Explant	MS (1962) supplemented with different PGRs	Response	References
Cassia angustifolia	Immature cotyledons		*% SE*	
		10 µM 2,4-D	83.90	Parveen and Shahzad (2014a, b)
		10 µM 2,4-T	10.26	
		10 µM IAA	05.86	
		10 µM IBA	00.00	
		10 µM NAA	30.30	
Petiveria alliacea	In vitro raised shoot-derived leaf segments		*% Response*	
		2.2 µM TDZ	Necrosis	Cantelmo et al. (2013)
		22.8 µM TDZ	Friable callus (33)	
		2.0 µM PIC	SE/compact callus (100)	
		20.0 µM PIC	SE/friable callus (100)	
		2.2 µM 2,4-D	SE/friable callus (100 %)	
		22.6 µM 2,4-D	SE/friable callus (100)	
Catharanthus roseus			*% SE*	
	Mature seeds	2.5 µM TDZ	18.0	Dhandapani et al. (2008)
		2.2 µM BA	25.6	
		2.4 µM IBA	75.6	
		2.5 µM BA + 5.3 µM NAA	82.2	
Corydalis yanhusuo			*Mean no. of SE per callus*	
	Tuber	0.5 mg/l BA	10.9	Sagare et al. (2000)
		0.5 mg/l Kn	07.5	
		0.5 mg/l Zea	09.0	

BA, Kn, Zea, NAA, TDZ, PIC, 2,4-D, IBA, MS medium

recorded on the percentage of plant regeneration from somatic embryos, i.e. the response varies from 74.44 to 85.56 %; the maximum percentage was observed in PCR cultivar. Akula et al. (2003) observed significant variation in the frequency of somatic embryogenesis from root segments in seven different genotypes of *Azadirachta indica*. Only four out of seven genotypes tested showed somatic embryogenesis that too with different frequencies. The highest response with 68 % frequency was observed in clone 5.6, while the lowest frequency (23 %) of somatic embryogenesis was exhibited by clone 20. Clones 10 and 11 did not exhibit somatic embryogenesis, but slight callus development was observed. No response (neither callus nor somatic embryos) was observed for clone 16. In clone 20, more than 50 % of explants showed callus induction, while induction of somatic embryos was observed in 23 % of explants. In alfalfa, among the three genotypes, A70–34 was found to be highly

Table 1.8 Effect of explant types on somatic embryogenesis of some medicinal plants

Plant and explant type	Medium	Type of embryogenesis	Response	References
Catharanthus roseus			*Mean no. of SE/culture*	
Hypocotyl	MS + 1 mg/L 2,4-D (ECIM); MS + 1 mg/L NAA + 1.5 mg/L BAP	Indirect	92.6	Dipti and Mujib (2014)
Root			00.0	
Leaf			00.0	
Stem			00.0	
Ochna integerrima			*Mean no. of shoots and SE*	
Shoot explant	MS + 15.0 μM TDZ	Indirect	48.2	Guohua Ma et al. (2011)
Leaf explant		Indirect	15.9	
Catharanthus roseus			*% SE*	
Mature embryo	MS + 7.5 μM TDZ	Direct	48.7	Dhandapani et al. (2008)
Hypocotyl		No response	00.0	
Cotyledon		No response	00.0	
Petiole		Indirect	06.0	
Shoot tip		Indirect	04.0	
Stem node		Indirect	04.0	
Piper nigrum			% SE	
Intact seeds	PGR-free SH medium	Direct	16.0	Nair and Gupta (2005)
In vitro germinated seeds		Direct	24.0	
In vitro abortively germinated seeds		Direct	32.0	
Unripened green fruits (zygotic embryo removed)		Direct	05.0	
Zygotic embryos		Direct	00.0	
Azadirachta indica			% SE	
Root	MS I (half-strength macrosalts + full-strength microsalts of MS medium + 1 g/l CH + 100 mg/l myo-inositol + 100 mg/l AdS + 100 mg /l glutamine)	Direct	72.0	Akula et al. (2003)
Nodal segment			66.2	
Leaf	MS I + 2.3 μM TDZ + 0.5 μM 2,4-D	Indirect	35.2	
Eleutherococcus sessiliflorus			% SE	
Hypocotyl	MS + 4.5 μM 2,4-D	Direct	45.0	Choi et al. (2002)
Cotyledon		Direct	33.0	
Root		Direct	08.0	

MS, 2, 4-D, TDZ, CH, AdS, SH, NAA, BAP

embryogenesis on MS I from root and nodal explants, wherein root exhibited better somatic embryogenesis (72 %) than nodal segments (66.2 %), while indirect embryogenesis (35.2 %) was observed from leaf explants of the same plant on TDZ and 2,4-D containing MS I medium (Table 1.8).

1.3.2.4 Effect of Liquid Culture System

Among other factors, liquid culture systems also have the profound effect on the induction and maintenance of somatic embryoids in many plant species. Suspension cultures have proven to be embryogenically uniform and can be maintained for a very long period. Also, embryogenic suspension cultures are an important source for the identification and examination of certain events in somatic embryo development. Liquid media were used from the beginning of research to study the developmental pathways of cells leading to somatic embryoid formation (Steward et al. 1958; Reinert 1958). Somatic embryo production through suspension cultures has also been used in medicinal plant species such as *Catharanthus roseus* (Kim et al. 2004) and *Plumbago roseus* (Silja et al. 2014). The use of bioreactors for the maintenance of embryogenic cell suspensions at large scale has been reported by various workers in different plant species (Bapat et al. 1990), sweet potato (Bienick et al. 1995), *Picea sitchensis* (Ingram and Mavituna 2000), etc. (Table 1.9).

1.3.2.5 Effect of Genotype

Genotype has a profound effect on the induction of somatic embryogenesis in various plant species. Although genotypic specificity regarding somatic embryogenesis has been reported in various plant species like wheat (Maës et al. 1996), melon (Yadav et al. 1996), maize (Close and Ludeman 1987), soybean (Parrott et al. 1989), cotton (Sakhanokho et al. 2001; Rao et al. 2006) and coffee (Molina et al. 2002), there is scarcity of literature on the genotype-dependent somatic embryogenesis and plant regeneration on medicinally important plants. Nair and Gupta (2005) reported that out of 15 genotypes (Jeerakamundi, Kalluvally, Karimunda, Kutching, Kuthiravally, Narayakodi, Neelamundi, Neyyattinkaramunda, Panniyur-1, Perambramunda, Sreekara, Subhakara, Thevanmundi, Thommenkodi and Vadakkan) of black pepper tested, 14 showed the embryogenic response while Malaysian cultivar ‘Kutching’ did not exhibit any somatic embryogenic response. Among the responded genotypes, ‘Karimunda’ exhibited the highest frequency of embryogenesis (28.0 %) with the formation of about 7.0 somatic embryos per explant.

Genotypic effect of three *Catharanthus roseus* cultivars Pacifica cherry red (PCR), Heatwave mix colour (HWMC) and Mediterranean Rose Red (MRR) on somatic embryogenesis through hypocotyl explants was elaborated by Yuan et al. (2011). They reported that similar responses of primary callus and embryogenic callus formation were observed in all the three cultivars, but a significant difference was

Table 1.9 Effect of liquid culture system on somatic embryogenesis of some medicinal plants

Plant	Initiation of embryogenic calli		Establishment of suspension culture	Result	References
	Explant	Medium			
Plumbago rosea	Leaf	MS + 2 mg/l 2,4-D + 1 mg/l NAA + 2.5 mg/l BA	1 g callus to liquid MS + 2 mg/l 2,4-D + 1 mg/l NAA + 2.5 mg/l BA	No. of SE (data not given	Silja et al. (2014)
Echinacea purpurea	In vitro grown leaf disc	MS + 1 μM TDZ	0.3 g slurry in 50 ml MS liquid media + 1.0 μM TDZ	No. of SE approx. 800	Jones et al. (2007)
Catharanthus roseus	Immature zygotic embryos	MS basal medium	1 g embryogenic callus in 20 ml of liquid MS + 4.52 μM 2,4-D medium; 5 ml of this suspension into 50 ml liquid MS + 4.52 μM 2,4-D	56.7 % conversion of SE to plantlet on solid MS basal medium	Kim et al. (2004)
Hylomecon vernalis	Petiole	B5 + 13.6 μM 2,4-D	1 g callus in liquid B5 medium + 4.52 μM 2,4-D; 5 ml of this suspension into50 ml B5 + 4.52 μM 2,4-D	70 % SE	Kim et al. (2003)
Acanthopanax koreanum Nakai	Internode	MS + 4.5 μM 2,4-D	Filtered cells (200 μl) in 30 ml MS liquid medium + 0.45 μM 2,4-D	≈350 SE	Choi et al. (1997)

B5, MS, 2,4-D, SE, NAA, BA, TDZ

Table 1.10 Effect of genotype on somatic embryogenesis (SE) of some potentially important medicinal plants

Plant and genotype	Explants used	Medium	Response	References
Catharanthus roseus			% Plant regenerated from SE	
cv. PCR	Hypocotyl	MSCP (MS 1962 + 150 mg/L CH + 250 mg/ L proline, + 30 g/ L sucrose + 3 g/L gelrite.)	85	Yuan et al. (2011)
cv. HWMC			78	
cv. MRR			74	
Piper nigrum			% SE	
cv. Jeerakamundi	Germinating seeds	PGR-free SH medium	11.0	Nair and Gupta (2005)
cv. Karimunda			28.0	
cv. Kutching			0.0	
cv. Sreekara			23.0	
Azadirachta indica			% SE	
A. indica 5.6	Root	MS 1 (half-strength macrosalts and full-strength microsalts of MS medium + 1 g/L CH + 100 mg /L myo-inositol + 100 mg /L AdS + 100 mg/L L-glutamine)	68.2	Akula et al. (2003)
A. indica 10			00.0	
A. indica 20			23.1	
A. indica 5.2			50	
Medicago sativa			Mean no. of SE	
cv. RA3	Ovary and petiole tissue	SH	50	Skokut et al. (1985)
cv. RA3 × falcata regen.			20	
cv. RA3 × falcata non-regen.			0	

embryogenic, R3 produced callus but not somatic embryoids and MK did not show any response (Hernandez-Fernandez and Christie 1989). In contrast to above studies, Franklin and Dias (2006) found that different genotypes (Helos, Topas, Elixir and Numi) of *Hypericum perforatum* responded similarly with no significant differences in somatic embryogenesis and plantlet production (Table 1.10).

1.3.2.6 Effect of Culture Room Conditions

Somatic embryogenesis is also regulated by culture room conditions. Environmental factors such as light intensity, temperatures, humidity, etc. are important determinants for the acquisition of embryogenic competence by the somatic cells (FehÈr 2008). Yang et al. (2013) reported that relatively high temperature was effective for induction of secondary SEs from hypocotyls of germinated primary somatic embryos of *Hovenia dulcis*. The maximum number (97.2) of secondary SEs was formed at 30 °C, whereas the lowest (5.7) was induced at 20 °C. Therefore, high

temperature stress can turn somatic cells into embryogenic cells. Although many species can form embryo in light as well as in darkness (Gingas and Lineberger 1989; Mikuła and Rybczyński 2001), promotion and inhibition of embryo by light are also well documented. Gingas and Lineberger (1989) reported greatest number of somatic embryos from explants incubated in light, whereas high irradiance inhibited embryogenesis in cotyledon cultures of soybean (Lazzeri et al. 1987). In *Gentiana tibetica* cultures maintained in light formed the first embryogenic centres in the fifth week, whereas embryogenesis was delayed for further 2 weeks in cultures maintained in the dark (Mikuła and Rybczyński 2001). The effect of alternating exposures to dark and light incubation conditions has also been examined in olive, wherein somatic embryogenesis only occurred from zygotic embryos that were first incubated in dark for 3 weeks and thereafter in light. Incubation only in light completely inhibited embryogenesis (Rugini 1988) (Table 1.11).

1.3.3 Root Induction in Microshoots

Rooting in regenerated microshoots is an important step in micropropagation, which is essential for the development of complete plantlets. It involves three distinct phases, namely, induction, initiation and expression (Kevers et al. 1997; De Klerk et al. 1999). In the absence of proper root system, plantlets will not be able to survive under external or ex vitro conditions, and losses at this stage have vast economic consequences (De Klerk 2002). Rooting can be induced via in vitro or ex vitro methods.

1.3.3.1 In Vitro Rooting

Strength of the MS medium played an important role in rooting of microshoots. It was observed that in *Cassia angustifolia*, full-strength MS medium without any auxin failed to induce rooting, while reducing the strength of MS medium to half proved to be beneficial (Parveen et al. 2012). Rooting on auxin-free MS basal medium has been reported by Reddy et al. (1998) in *Gymnema sylvestre* and Pyati et al. (2002) in *Dendrobium macrostachyum*, while superiority of half-strength MS in rooting over full-strength MS medium has also been well documented in the literature (Nabi et al. 2002; Parveen et al. 2010; Shahzad et al. 2012; Sharma et al. 2014). IBA proved to be more efficient for rooting than other auxins in number of plants such as *Cunila galoides* (Fracaro and Echeverrigaray 2001), *Embelia ribes* (Raghu et al. 2006), *Clitoria ternatea* (Shahzad et al. 2007), *Cassia siamea* (Parveen et al. 2010) and *C. sophera* (Parveen et al. 2010). Another reason for being more potent auxin is that IBA is comparatively lesser degraded by autoclaving than IAA and is generally considered to be more stable in the light than IAA, which is rapidly photo-oxidized (Nissen and Sutter 1990; Epstein and Müller 1993; De Klerk et al. 1999).

Table 1.11 Effect of culture room conditions on SE induction of some selected medicinal plants

Plant name	Explant/medium used	Culture room conditions	Response	References
Hovenia dulcis		*Temperature (°C)*	*No. of secondary SEs/explant*	
	Mature seeds/ MS agar medium	20	5.7	Yang et al. (2013)
		25	65.3	
		30	97.2	
Eleutherococcus senticosus		*Temperature (°C)*	*Growth ratio of embryos ({harvested dry weight (g) – inoculated dry weight (g)}/ inoculated dry weight (g) of the inocula)*	
	Young leaves/ MS + 1 mg/L 2,4-D	12	8.62	Shohael et al. (2006a)
		18	12.34	
		24	16.61	
		30	7.81	
Eleutherococcus senticosus		*Light quality*	*Growth ratio of embryos ({harvested dry weight (g) – inoculated dry weight (g)}/ inoculated dry weight (g) of the inoculums)*	
	Young leaves/ MS + 1 mg/L 2,4-D	Dark	19.05	Shohael et al. (2006b)
		Fluorescent	19.81	
		Blue	17.77	
		Red	15.22	
		Blue + far red (1:1)	19.11	
Gentiana tibetica	Cotyledons/ MS + 0.5 mg/L 2,4-D + 1.0 mg/L Kn	Dark/light	Callus proliferation: 60–70 %/40 %	Mikula and Rybczynski (2001)
G. pannonica		Dark/light	Callus proliferation: 60–70 %/40 %	
G. cruciata		Dark/light	Callus proliferation: 40 %/30 %	

Phloroglucinol (PG), a phenolic compound, is responsible for the suppression of peroxidase activity in the culture and thus protects the endogenous auxin from peroxidase-catalyzed oxidation which facilitates healthy root formation (De Klerk et al. 1999; Parveen et al. 2012). The promotive effect of PG on rooting was identified in several plant species including *Prunus avium* (Hammatt and Grant 1996), *Malus pumila* (Zanol et al. 1998), *Decalepis hamiltonii* (Giridhar et al. 2005) and *Pterocarpus marsupium* (Husain et al. 2007, 2008).

The gelling substance used in rooting medium also had a great impact on in vitro rooting (Parveen and Shahzad 2014a, b). The superiority of liquid medium in rooting has been observed in different plant species (Gangopadhyay et al. 2002, 2004). The filter paper bridge/support provided in liquid medium gave better anchorage owing to its porosity that facilitated better absorption throughout its surface area. Faisal et al. (2006) reported that rooting medium solidified by agar was more suitable than phytagel or gelrite and provided more branched and thicker roots in *Mucuna pruriens*.

1.3.3.2 *Ex Vitro* Rooting

In vitro rooting in *Cassia angustifolia* involves several problems like necrosis of shoot tips and yellowing or abscission of leaves on transferring to rooting media. The formation of callus at cut end of the microshoots also prevented the development of roots in auxin-supplemented medium (Parveen and Shahzad 2011). Thus, to rectify the problems of in vitro rooting, an alternative method (ex vitro rooting) was adopted to induce rooting in *C. angustifolia*. Through ex vitro rooting technique, necrosis and leaf abscission have been minimized considerably, and healthy developmental pattern was observed. Pandeya et al. (2010) in *Clitoria ternatea* also reported *ex vitro* rooting through pulse treatment with 250 mg/l IBA for half an hour.

Bozena (2001) suggested that the plantlets of strawberry developed after ex vitro rooting have better root system than the ones raised through in vitro rooting. Rooting in the external environment is an aid for simultaneous hardening and acclimatization of plantlets and decreases the micropropagation cost as well as the time from laboratory to field conditions (Pruski et al. 2000). Ex vitro rooting proved to be more advantageous over in vitro rooting, as the latter requires utmost care during planting and also more labour and time. This is in corroboration with the earlier studies in several other plant species such as *Lagerstroemia parviflora* (Tiwari et al. 2002), *Prunus fruticosa* (Kris et al. 2005), *Celastrus paniculatus* (Martin et al. 2006), *Aegle marmelos* (Raghu et al. 2007), *Holarrhena antidysenterica* (Mallikarjuna and Rajendrudu 2007) and *Siraitia grosvenorii* (Yan et al. 2010) *Tecomella undulata* (Shaheen and Shahzad 2015).

1.3.4 Acclimatization of Plantlets in Natural Environment

The success of any micropropagation protocol depends on the acclimatization of regenerated plantlets in the external environment at low cost and with high survival rate. During this period of transition, from in vitro to ex vitro conditions, plants have to overcome many adverse conditions as they were cultured under aseptic conditions, with low light intensity on medium containing ample sugar and nutrients to allow heterotrophic growth in the atmosphere of high relative humidity. These conditions result in the plantlets with altered morphology, anatomy and physiology (Kozai et al. 1991; Pospíšilová et al. 2007). Therefore, after ex vitro transplantation, plantlets usually need few weeks of acclimatization and gradually overcome these inadequacies to adapt in the external environment. The survival of plantlets during acclimatization also depends on the use of suitable planting substrate.

After successful acclimatization, in vitro raised plantlets are transferred to earthen pots containing sterilized soil and manure (1:1) and kept under greenhouse for 2 weeks and then finally transferred to the field. These plants do not show any detectable variation in morphological or growth characteristics when compared to the control plants.

1.4 Summary and Future Prospects

Success of plant biotechnology is dependent on regeneration of intact plants following genetic modification, and it has been achieved using plant tissue culture technology. Plant tissue culture helps regenerating a whole plant from a small tissue or a cell, in a suitable culture medium under controlled environmental conditions, and has become an integral part of plant breeding also. It has been effectively used for mass production of elite clones of crop plants where other viable forms of propagation are not available. It has also been successfully used in agriculture-related business. Various types of fruits, flowers, medicinal plants and even trees have been successfully propagated through plant tissue culture.

Thus, plant tissue culture represents the most promising areas of application at present time and gives an outlook into the future. Quality control, however, is also very essential to assure high-quality plant production. The selection of explant source, disease-free material and authenticity of variety are some of the critical parameters which should be evaluated to ensure the quality of the plants produced through this technology.

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Chapter 2
Plant Tissue Culture: Applications in Plant Improvement and Conservation

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Abstract Plant tissue culture is a significant contribution in micropropagation of ornamental and forest trees, production of pharmaceutically interesting compounds and plant breeding for improved nutritional value of staple crop plants as well as in the improvement of tree species. Plant tissue culture can provide high-quality planting material for the fruits, vegetables and ornamental plants and forest tree species throughout the year, irrespective of season and weather, thus opening new opportunities to producers, farmers and nursery owners. The biotechnological approaches like haploid induction, somaclonal variation, etc. to improve traits are also its important applications. Plant tissue culture is a noble approach to be used in bioproduction, bioconversion or biotransformation of the valuable secondary products for large-scale production and biosynthetic studies.

Plant tissue culture has been routinely done for conservation of germplasm to be used for improving secondary metabolite production and agronomic traits of crops to increase yields. Production of artificial seeds has unravelled new vistas in in vitro plant biotechnology, such as large-scale clonal propagation, delivery of clonal plantlets, germplasm conservation and breeding of plants in which propagation through normal seeds is not possible.

2.1 Introduction

Tissue culture has been successfully used to improve the vigour and vitality of the plants. The technique has been used to create new genetic variation in the breeding lines, often via haploid production, to be used by plant scientists. Earlier, to create genetic diversity and manipulate genetic variability of plants, controlled pollination of plants was done. The specific cross would result in a new generation that

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performed better in the field than either of the parents or the progeny of subsequent generations, i.e. the expression of heterosis through hybrid vigour. Genetic manipulation of genome has been broaden with the appropriate application of plant tissue culture and molecular technique, thereby considerably reducing the dependency on the time-consuming classical breeding mode of pollination and cross-fertilization.

In self-pollinated species, homozygosity is required in cultivars to be released for general cultivation, so that no segregation occurs in field. The use of haploids (H) and double (DH) haploids developed through tissue culture drastically reduces the time for precise and efficient selection for desirable traits (Basu et al. 2011). The DH lines can directly be released as varieties for general cultivation, used as parents in breeding programmes, to develop inbred lines for hybrid seed production in case of cross-pollinated species or stored as germplasm lines (Touraev et al. 2009). Moreover, fertile DH lines have been developed in some species having genetic barriers in inbreeding or self-incompatibility hence, helps in breaking the genetic barriers (Grauda et al. 2010). Somaclonal variation in tissue culture has also been used as alternative sources for obtaining new variants with desirable agronomic traits (Kearsey 2002).

Tissue culture has also been successfully used for production of secondary metabolites. These metabolites accumulate in plants as part of defence response against pathogenic attack or wounding. Their biosyntheses are triggered and activated by elicitors, the signal compounds of plant defence responses. The production of secondary metabolites has been enhanced in a number of plant species by employing the techniques of elicitation and biotransformation. The use of bioreactor in plant tissue culture has greatly increased the multiplication and growth of cultures with reduced energy, labour and space requirement. Thus, the ability to accelerate the conventional multiplication rate could be of great benefit in situation where a disease or some climatic disaster wipes out crops. Further, the loss of genetic resources is very common where the germplasm is held in field gene banks. Cryopreservation and in vitro storage could be used in problems inherent in field gene banks, providing a secure duplicate collection. The germplasm could be stored for longer durations and allow future generations to access it. Thus, the genetic biodiversity through creation and conservation could be exploited for improving secondary metabolite production and agronomic traits of crop to increase yields.

2.2 Haploids and Double Haploids

In vitro production of haploids can solve some problems in genetic studies since gene action is readily manifested due to a single allelic gene present in chromosome of entire genome. Haploid breeding technique usually involves only one cycle of meiotic recombination. They are smaller and exhibit a lower plant vigour compared to donor plants and are sterile due to the inability of their chromosomes to pair

during meiosis. In order to propagate them through seed and to include them in breeding programmes, their fertility has to be restored with spontaneous or induced chromosome doubling. The obtained double haploids (DH) are homozygous at all loci and can represent a new variety (self-pollinated crops) or parental inbred line for the production of hybrid varieties (cross-pollinated crops). Haploids provide a relatively easier system for the induction of mutations and mutants with resistance to disease are of prime importance in crop improvement. The chromosomal instability in haploids also makes them potential tools for introduction of desired genes during wider crossing programmes and reducing the overall time in developing the new variety. Single recombination event, however, is unable to release all potential variations, so haploids are usually insufficient for the improvement of polygenically controlled agronomic traits. In double haploids, homozygosity is achieved in one generation, eliminating the need for several generations of self-pollination and saving substantial time.

Haploid and double haploid plants can be produced by a variety of methods. A naturally occurring haploid from *Datura stramonium* was first described by Blakeslee in 1922, and, since then, there have been many reports of other naturally occurring haploids (Dunwell 2010). The disadvantage of spontaneous haploids is the low frequency in which they occur making this method ineffective for breeding purposes or basic research. Haploids can also be produced by wide crosses (interspecific and intergeneric) followed by chromosome elimination (Rines et al. 1996) or parthenogenesis (Sestili and Ficcadenti 1996). The most common method to produce haploid and double haploid plants is in vitro culture of male (i.e. microspores and anthers) or female (i.e. ovules or ovaries) gametophyte, termed as androgenesis or gynogenesis, respectively. When embryo or calli are produced from the gametophyte, these can be regenerated to haploid and double haploid plants. The anther culture technique was successfully used to establish both haploid and diploid somatic cell lines of pollen plants in wheat and maize.

Androgenesis via anther culture was first described by Guha and Maheshwari (1964) in *Datura*. Most of early work focused on anther culture. However, with improvement and refinements of the technique, isolated microspores from the anthers prior to culture eliminate the chance that anther tissue (i.e. somatic tissue) can regenerate plants, which is undesirable. Gynogenic methods for haploid production have been demonstrated for at least 24 species (Bohanec 2006). These methods are usually less efficient than androgenesis, and, therefore, fewer studies have been carried out in gynogenesis. Although gynogenic methods are beneficial when plants do not respond to androgenic methods, there is a problem with regenerating albino plants from anther culture, or the donor plants are male sterile.

Induced microspore can follow two routes of development to form a plant: direct embryogenesis similar to zygotic embryo development (i.e. globular, heart shaped, torpedo and cotyledonary stages) or indirectly through a callus phase which can be observed in several of medicinal plants. In a few case, both developmental pathways are observed and can occasionally be manipulated to favour embryos or callus. For

examples, two different media formulations differing in type and concentration of growth regulators are given for *Digitalis obscura*, one for the production of calli and one for the production of embryos (Perez-Bermudez et al. 1985). After developing cotyledonary embryos or calli, they can be removed from the anther/microspore culture media to a different media for the regeneration of plants. Regeneration usually takes place in the light on solid media.

In regenerated plants, ploidy level can be determined by counting chromosomes or by using a flow cytometry. In some species, the rate is very high for spontaneous chromosome doubling, and this saves the added step of chromosome doubling agents to produce fertile, homozygous, doubled haploid plants. For those species with a low frequency of spontaneous doubling, chromosome doubling agents like colchicine or trifluralin will have to be used.

There are also many original articles, books and review articles that have been published on double haploidy methodology via androgenesis or gynogenesis (Chen et al. 2011; Ferrie and Caswell 2011; Maluszynski et al. 2003).

2.2.1 Factors Affecting Haploid Induction

The following factors affect haploid induction:

1. Donor plant genotype
2. Physiological condition of donor plants (i.e. growth at lower temperature and high illumination)
3. Developmental stages of gametes, ovules and microspores
4. Pretreatment (i.e. cold treatment of inflorescence prior to culture, hot treatment of cultured microspores)
5. Culture medium composition
6. Physical factors during the tissue culture period (light, temperature and humidity)

2.2.2 Doubled Haploids in Medicinal Plants

There has been little work conducted on double haploids in medicinal plants compared to agronomically important crops. There are, however, reviews on the induction of double haploids in nutraceutical species (Ferrie 2007, 2009; Ferrie et al. 2005) providing list of species in which double haploidy methods have been attempted and have resulted in calli, embryos or plants (Table 2.1).

Table 2.1 List of nutraceutical/medicinal plant species in which double haploidy responses has been observed

Species	Common name	Method	Result	Media for callus/embryo induction	References
Aconitum carmichaelii	Chinese aconite	A	C	MS + 5 mg/L 2,4-D + 1 mg/L Kn + 3 % sucrose	Hatano et al. (1987)
Allium cepa	Onion	G	P	B5 + 1–2 mg/L 2,4-D + 1–2 mg/L BA + 7.5 % sucrose + 0.7 % agar	Sulistyaningsih et al. (2006)
Angelica archangelica	Angelica	IMC	C	NLN + 13%sucrose	Ferrie et al. (2005)
Azadirachta indica	Neem	A	C,P	MS + 1 μM 2,4-D + 1 μM and 5 μM BA + 9 % sucrose	Chaturvedi et al. (2003)
Azadirachta indica	Neem	G	C,P	MS + 1 μM 2,4-D + 5 μM BA + 9 % sucrose; MS + 0.5 μM 2,4-D	Srivastava et al. (2009)
Borago officinalis	Borage	IMC	C	NLN + 15–25 % sucrose.	Ferrie et al. (2005)
Calendula officinalis	Marigold	IMC	C,P	R92.01 + sucrose +24 °C dark	Ferrie and Caswell (2011)
Camellia sinensis	Tea	G	C	MS + 8.5 μM BA + 4.5 μM 2,4-D	Hazarika and Chaturvedi (2013)
Carica spp.	Papaya	A	E, P	½ MS + full strength Na-Fe EDTA + 2.0 mg/L NAA + 1.0 mg/L BA + 60 g/L sucrose	Azad et al., (2013)
Carum carvi	Caraway	A	E, P	MS + 0.1 mg/L NAA + 0.1 mg/L 2,4-D + 0.5 mg/L BA + 15 % CM + 2 % sucrose	Smykalova et al. (2009)
Fagopyrum esculentum	Buckwheat	A	C,P	MS + 2.5 mg/L BA + 0.5 mg/L IAA + 90 g/L maltose	Bohanec et al. (1993)
Fagopyrum esculentum	Buckwheat	G	C,P	MS + 2.5 mg/L BA + 0.5 mg/L IAA + 90 mg/L sucrose	Bohanec (1997)
Foeniculum vulgare	Fennel	A	C	MS + 1 mg/L 2,4-D	Matsubara et al. (1995)
Gentiana triflora	Clustered gentian	A	E, P	½ NLN + 30 g/L sucrose, ½ MS + 0.25 % gellen gum + 3 % sucrose; NN + 0.7 mg/L NOA + 1.2 mg/L BA + 3 % sucrose	Doi et al. (2010); Pathirana et al. (2011)
Gentiana triflora	Clustered gentian	G	E,P	½ NLN + 100 mg/L sucrose, ½ MS + 1 mg/L GA_3 + 1.0 % agar + 3 % sucrose	Doi et al. (2011)
Gingko biloba	Gingko	IMC	E	BN + 11.40 μM IAA + 0.93 μM Kn	Laurain et al. (1993a)

(continued)

Table 2.1 (continued)

Species	Common name	Method	Result	Media for callus/embryo induction	References
Gingko biloba	Gingko	G	E, P	MT + BA + NAA + glutamine, BN + CM	Laurain et al. (1993b)
Hepatica nobilis	Sharp-lobed hepatica	A	E, P	NN + 1 % AC at 35 °C, NN at 15 °C	Nomizu et al. (2004)
Hieracium pilosella	Mouse-ear hawkweed	A	C,P	MS + 0.5 mg/L 2,4-D + 200 mg/L glutamine + 3 % sucrose, MS + B5 vitamins + 1.0 mg/L BA + 3 % sucrose	Bicknell and Borst (1996)
Hyoscyamus muticus	Henbane	G	E,P	MS + 1.0 mg/L 2,4-D + 0.5 mg/L BA, MS + 0.05 mg/L NAA + 0.5 mg/L BA	Chand and Basu (1998)
Levisticum officinale	Lovage	A	E, P	MS + 10.74 μM NAA + 9.05 μM 2,4-D + 8.80 μM BA + 6 % glucose	Wang et al. (2014)
Matricaria recutita	Chamomile	IMC	C, P	R92.01 + maltose	Ferrie and Caswell (2011)
Mentha spp.	Mint	A	C	MS + 0.5 mg/L BA + 0.5 mg/L NAA	Van Eck and Kitto (1990)
Oenothera hookeri	Evening primrose	A	C, P	MS + 2.0 mg/L 2,4-D + 2.0 mg/L BA + 3 % sucrose	Martinez and de Halec (1995)
Panax ginseng	Ginseng	A	C,P	MS + 2 mg/L 2,4-D + 2 mg/L IAA + 1 mg/L IBA + 6 % sucrose	Du et al. (1987)
Panax quinquefolius	American Ginseng	A	C, P	N6 + 5 mg/L 2,4-D + 1 mg/L Kn + 3 % sucrose	Shao and Li (1986)
Papaver somniferum	Opium popy	A	C, P	MS + 2 mg/L 2,4-D + 0.5 mg/L + IAA + 0.5	Dieu and Dunwell (1988)
Physalis ixocarpa	Husk tomato	A	E, P	NN + 5 mg/L IAA + 0.2 mg/L Kn	Bapat and Wenzel (1982)
Psoralea corylifolia	Scurf pea	G	C, P	MS + 10.7 μM NAA + 4.5 μM 2,4-D, MS + 2.2–8.8 μM BA	Chand and Sahrawat (2007)
Pimpinella anisum	Anise	IMC	E, P	NLN + 25–30 % sucrose	Ferrie et al. (2011)
Salvia sclarea	Clary sage	A	C	MS	Bugara (1986)
Saponaria vaccaria	Cow cockle	IMC	E, P	NLN + 15 % sucrose	Kernan and Ferrie (2006)

Growth regulators and additives: *AC* activated charcoal, *BA* 6-benzylamino purine, *2,4-D* 2,4-dichlorophenoxyacetic acid, *CH* casein hydrolysate, *CM* coconut milk, *IAA* indole acetic acid, *1BA* indole butyric acid, *Kn* kinetin, *NAA* napthalene acetic acid, *NOA* naphthoxyacetic acid
Response: *A* anther culture, *G* gynogenesis, *IMC* isolated microspore culture, *C* callus, *E* embryo, *P* plant

2.3 Cell/Callus Culture

Plant cells are totipotent, which means that each plant cell in culture retains complete genetic information and hence is able to produce the range of chemicals found in the parent plant. Ahmed et al. (2009) studied in vitro production of gymnemic acid (an antidiabetic) pentacyclic triterpenoid isolated from the leaves of *Gymnema sylvestre* through callus culture under abiotic stress conditions. The production of gymnemic acid was significantly higher in callus treated with 2, 4-dichlorophenoxyacetic acid (2, 4-D) and kinetin (KN). The blue light increased gymnemic acid accumulation up to 4.4-fold as compared with fluorescent light treatment.

Jain et al. (2012) reported β-sitosterol and caffeic acid production in *Sericostoma pauciflorum* Stocks (family Boraginaceae) which are used against cancer and diabetes and are known to be health promoters. Callus cultures have been established from the stem explants on Murashige and Skoog (MS) medium supplemented with different growth hormones, viz. kinetin (Kn), indole 3-acetic acid (IAA) and indole 3-butyric acid (IBA). At 6 weeks of age, these calli were harvested, dried and extracted successively in petroleum ether, methanol and water. Extracts were dried, weighed (%) and analysed for their bioefficacies. The bioactive secondary metabolites, isolated from culture tissue of a 6-week-old callus, were identified by colour reaction, TLC behaviour and IR spectrum (Jain et al. 2012).

Recently, Ali and Tariq (2013) studied the secondary metabolite production in callus cultures of *Momordica charantia* cultivar Jaunpuri. A combination of BAP and 2,4-D proved to be the most suitable among several PGR combinations tested for the callus induction through in vitro-derived explants. The analysis of callus and the tissues from field-grown plants by using GC-MS (gas chromatography/mass spectrometry) resulted that mother tissues contain more secondary metabolites (Table 2.2).

Table 2.2 Bioactive secondary metabolites from plant cell culture (in chronological order)

Plant	Active ingredient	Culture type	References
Corydalis ophiocarpa	Isoquinoline alkaloids	Callus	Iwasa and Takao (1982)
Corydalis ophiocarpa	Isoquinoline alkaloids	Callus	Iwasa and Takao (1982)
Coffea arabica	Caffeine	Callus	Waller et al. (1983)
Citrus sp.	Naringin, limonin	Callus	Barthe et al.(1987)
Glycyrrhiza glabra var. *glandulifera*	Triterpenes	Callus	Ayabe et al. (1990)
Ginkgo biloba	Ginkgolide A	Suspension	Carrier et al. (1991)
Catharanthus roseus	Indole alkaloids	Suspension	Moreno et al. (1993)
Dioscorea doryophora Hance	Diosgenin	Suspension	Huang et al. (1993)
Ephedra spp.	L-ephedrine, D-pseudoephedrine	Suspension	O'Dowd et al. (1993)
C. roseus	Catharanthine	Suspension	Zhao et al. (2001)
Nothapodytes foetida	Camptothecin	Callus	Thengane et al. (2003)

2.4 Cell Suspension Culture

In cell suspension cultures, individual cells or groups of cells grow in a liquid medium. These are relatively homogeneous population of cells that can be easily exposed to nutrients. Cell suspensions constitute a good biological material for studying biosynthetic pathways. Indeed, compared to callus cultures, they allow the recovery of large quantities of cells from which enzymes can be more easily isolated (Dougall 1981). These biosynthetic studies enable the researcher to spot limiting enzyme activities (or genes) in the production of valuable metabolites.

Liu and Saxena (2009) reported flavonoid production in *Saussurea medusa* cell suspension cultures. To induce callus from leaf explants, a combination treatment of MS + BA (0.5 mg/L) + NAA (2 mg/L) + sucrose (30 g/L) + agar (5 g/L) was found to be the most suitable. A fine cell suspension was established from the induced light-yellow calli in the MS liquid medium with 30 g/L sucrose, 0.5 mg/L BA and 2.0 mg/L NAA for biosynthesis of flavonoids. The highest dry weight and flavonoid production reached 17.2 g/L and 607.8 mg/L, respectively after 15 days (Liu and Saxena (2009) (Table 2.2).

2.5 Organ Culture

(a) *Hairy root culture*: Hairy roots (HRs) are differentiated cultures of transformed roots generated by the infection of wounded plants with *Agrobacterium rhizogenes*. This pathogen causes the HR disease leading to the neoplastic growth of roots that are characterized by high growth rate in hormone-free media and genetic stability. HR produces the phytochemicals similar to that produced by wild-type organs. High stability and productivity features allow the exploitation of HRs as valuable biotechnological tool for the production of plant secondary metabolites. In addition, several elicitation methods can be used to further enhance their accumulation in both small- and large-scale production. However, in the latter case, cultivation in bioreactors should be required to optimize. HRs can also be utilized as a biological form for the production of recombinant proteins, hence holding additional potential for industrial use. HR technology has been strongly improved by increased knowledge of molecular mechanisms underlying their development.

Strycharz and Shetty (2002) used this approach in *Mentha pulegium* clonal line MPH-4 to increase the production of total plant phenolic compounds and guaiacol peroxidase. Enhancement of phenolic production by A. rhizogenes was more apparent, when explants from *M. pulegium* were also treated with polymeric dye R-478.

(b) *Shoot culture*: Coste et al. (2011) studied the effect of plant growth regulators and elicitors on the production of secondary metabolites in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*. Culture of shoots on MS medium supplemented with BA (0.4 mg L^{-1}) or Kin (0.4 mg L^{-1}) enhanced

production of hypericins in *H. maculatum* and hyperforin in *H. hirsutum*. Cultivation of *H. maculatum* on modified Murashige and Skoog (MS) medium containing 10 mM NH_4^+ and 5 mM NO_3^- medium resulted in approximately twofold increased production of hypericins compared to controls, and the growth of *H. hirsutum* shoots on the same medium led to a 6.16-fold increase in hyperforin production. Of the two elicitors, SA was more effective in stimulating the accumulation of hypericins. At 50 μM, SA enhanced the production of hypericin (7.98-fold) and pseudohypericin (13.58-fold) in *H. hirsutum* and, at 200 μM, enhanced the production of hypericin (2.2-fold) and pseudohypericin (3.94-fold) in *H. maculatum.*

(c) *Tissues and organs*: Garlic extract contains alliin (S-allyl-L-cysteine sulfoxide), an organosulphur compound that contributes to its therapeutic value and pharmacological importance. Alliin production in plant organs (leaf and root), plantlet, callus (non-embryogenic and embryogenic) and embryo (proliferated, matured and germinated) grown under in vitro conditions was evaluated after 8 weeks. Highest alliin content was recorded in leaves. Clove callus and germinated embryos also showed alliin production. Evaluation of alliin content of in vitro-grown tissues both in normal (control) and sulphur supplemented conditions (4, 8, 16, 32 mg L^{-1}) showed that sulphur treatment at 16 mg L^{-1} as gypsum ($CaSO_4$) significantly enhanced the production of alliin content in in vitro-grown tissues and organs (Nasim et al. (2010).

2.6 Biotransformation

Biotransformation can be defined as a process through which the functional groups of organic compounds are modified either stereo- or regiospecifically by living cultures, entrapped enzymes or permeabilized cells to a chemically different product (Ramachandra and Ravishankar 2002). Cell suspension cultures, immobilized cells, enzyme preparations and hairy root cultures can be applied for the production of food additives or pharmaceuticals by biotransformation process. György and Hohtola (2009) studied the production of cinnamyl glycosides from the compact callus of *Rhodiola rosea* through the biotransformation of cinnamyl alcohol.

2.7 Elicitation

Elicitation strategies induce plants to synthesize phytoalexins at elevated levels in plant cell cultures, thereby reducing the process time to attain high product concentrations and increased culture volumes (Barz et al. 1988). Actually, elicitors are signals triggering the formation of secondary metabolites.

Korsangruang et al. (2010) studied the effects of abiotic and biotic elicitors on growth and isoflavonoid accumulation in *Pueraria candollei* var. candollei (PC) and *P. candollei* var. mirifica (PM) cell suspension cultures. The two plant varieties

exhibited different growth responses and varied isoflavonoid accumulation after the addition of elicitors. Copper sulphate, methyl jasmonate (MeJA) and yeast extract did not significantly affect the growth of either plant variety, whereas oligosaccharide and the biotic elicitors used in this study (i.e. 50 mg L^{-1} chitosan and all concentrations of laminarin) suppressed the growth of PM. The addition of MeJA to the medium principally induced an effect on the isoflavonoid content in both PM and PC, with 2.0 μM MeJA inducing the highest isoflavonoid content, as indicated by the induction index-4.41 in PM and 9.62 in PC cells on the 12th and ninth day of culture, respectively. A maximum total isoflavonoid content of 40.49 mg g^{-1} dry weight was achieved in PM 21 days after elicitation with 2.0 μM MeJA. LAM elicited the PM cell suspension culture to produce puerarin, which was not found in the unelicited culture (Korsangruang et al. 2010).

2.8 Secondary Metabolite Production Through Plant Tissue Culture

Plants produce various chemicals that are useful as medicines, food, flavouring compounds and cosmetics, for example, phytochemicals like L-DOPA, morphine, codeine, reserpine, vincristine, vinblastine and taxol are used as medicines. Often the source plants are cultivated in tropical or subtropical, geographically remote areas, which are subjected to political instability, drought, disease and changing land use patterns as well as other environmental factors. In addition, the long cultivation periods between planting and harvesting make selection of high-yielding strains difficult, thus resulting in expensive drugs. Cultivation periods may range from several months to decades for the taxol-yielding tree, *Taxus brevifolia*. In spite of these difficulties and costs, the extraction of medicines from cultivated plants or plants in the wild continues due to the lack of credible alternatives (DiCosmo and Misawa 1995).

In the 1930s, the first in vitro cultures were established (White 1934; Gautheret 1939), and this was followed by a period of development of culture media and cultivation methods (Street 1977). The low yields of secondary metabolites in suspension cultures clearly were a bottleneck for commercialization. In these early efforts, plant cells in culture were treated in direct analogy to microbial systems, with little knowledge of plant cell physiology and biochemistry or the influence of bioreactor operation on the physiologic state of such systems. In 1982, at least 30 compounds were known to accumulate in plant culture systems in concentrations equal to or higher than that of the plant (Staba 1982). Advances in the area of cell cultures for the production of medicinal compounds have made possible the production of a wide variety of pharmaceuticals such as alkaloids, terpenoids, steroids, saponins, phenolics, flavonoids, amino acids and new therapeutics.

Plant tissue culture technology has been proved as a fruitful technique for the production of plant chemicals. Below are some advantages of secondary metabolite production through plant cell/tissue culture, rather than in vivo in the whole crop plant (Table 2.3).

Table 2.3 Transgenic cultures and plant systems used to produce secondary metabolites

Species	Uses	Transformation method	Marker gene used	Resulting transgenic tissue	References
Catharanthus roseus	Anticancer	*A. rhizogenes*	*gfp*	Hairy root	Rizvi et al. (2013)
Dracocephalum kotschyi	Against stomach and liver disorder	*A. rhizogenes*	*rolB*	Hairy root	Sharafi et al. (2014)
Gentiana scara	Used in gastric infection	*A. rhizogenes*	No marker gene selected	Hairy root	Huang et al. (2014)
Helicteres isors	Source of diosgenin	*A. rhizogenes*	*rolB*	Hairy root	Kumar et al. (2014)
Pterocarpus marsupium	Cytotoxic, antiproliferative	*A. tumefaciens*	*gus gene*	Shoot	Radhika et al. (2013)
Maackia amurensis	Hepatoprotective effect	*A. tumefaciens*	*rolC*	Callus	Grishchenko et al. (2013)
Rauwolfia serpentina	CNS disorder treatment, hypertension, insomnia	*A rhizogenes*	*crtdc*	Hairy root line	Mehrotra et al. (2015)
Tribulus terrestris	In sexual dysfunction	*A. rhizogenes*	*rolB*	Hairy root	Sharifi et al. (2012)
Leptadenia pyrotechnica	Medicinal use	*A. tumefaciens*	*gus/gfp*	Hypocotyls	Dutta et al. (2013)
Picrorhiza kurroa	Liver ailments, dyspepsia	*A. tumefaciens*	*gfp/hpt*	Leaf	Bhat et al. (2012)
Terminalia bellerica	Antimalarial and antibacterial	*A. tumefaciens*	*nptII/gus*	Cotyledon	Dangi et al. (2012)
Centaurea montana	Anticancer	*A. tumefaciens*	*ipt*	Leaf	Wissam et al. (2011)
Cuminum cyminum	Medical used	Microprojectile bombardment	*hpt II*	Embryo callus	Singh et al. (2010)
Ricinus communis	Castor oil	Particle gun-mediated	*gus/hptII*	Embryo callus	Sailaja et al. (2008)
Rauvolfia micrantha	Antiarrhythmic	*A. rhizogenes*	No marker gene selected	Plant	Sudha et al. (2003)

2.9 Somaclonal Variation and Crop Improvement

In nature, the process of recombination leads to the genetic diversity and variability within a population which is governed by several factors, viz. natural selection, mutation, migration and population size. Somaclonal variation is defined as variation originating in cell and tissue cultures, and the individuals are referred as "somaclone" (Larkin and Scowcroft 1981). Under in vitro condition, the process of growth, development and morphogenesis happens under the influence of exogenous supply of growth regulators. The continuous presence of higher doses of PGRs influenced the mitotic activities which often induced spontaneous variations in daughter cells. Thus, the regenerated plants from such types of tissues derived from organ culture, protoplast culture and callus culture may show genotypic and phenotypic variations. All or some individual could be differing from the donor plants. Both genetic and epigenetic changes have been reported; however majority show epigenetic changes. The variability generally occurred due to the culture room condition and various types of imposed stresses. Hence, the temporary changes are reversible and the permanent changes are heritable in nature.

The term somaclonal variation is now universally accepted to represent heritable variations arising in tissue culture. Visible pre-existing variations such as found in chimeric tissues could theoretically be cultured separately and later manifest themselves phenotypically in somaclones. These may not necessarily represent variations arising during tissue culture. The term should, therefore, be restricted to variations that were not visible to the naked eye during the culture initiation stage. In discussing somaclonal variation, both the negative and positive effects need to be treated in parallel. This is due to the potential induced variation in crop improvement as much as it is essential to detect and eliminate variants at early stages to minimize loss. Table 2.4 is showing the examples of plants with incidence of somaclonal variation, possible causes and their detection methods.

2.10 Synthetic Seed Technology

The first official definition of synthetic seeds (or artificial seeds or synseeds) was as "an encapsulated single somatic embryo, i.e., a clonal product that could be handled and used as a real seed for transport, storage and sowing, and that, therefore, would eventually grow, either *in vivo* or ex vitro, into a plantlet" (Murashige 1977). As per the definition proposed by Murashige, the researchers interested in synthetic seed technology restrict their effect in those plant species which were significantly responsive for somatic embryogenesis. Later in 1987, Bapat et al. expanded the horizon of synthetic seed research by encapsulating the in vitro-derived propagule other than the somatic embryoid, for the production of synthetic seeds. Because of the increased popularity of non-embryoid propagules encapsulation, Aitken-Christie et al. (1995) proposed a new definition of synthetic seed as "artificially encapsulated

Table 2.4 List of plants with incidence of somaclonal variation, possible causes and their detection methods

Plant	Common name	Source of variation	Detection method	References
Fragaria	Strawberry	BA	Morphology, RAPD	Biswas et al. (2009)
Freesia hybrid klatt	Freesia	Callus culture	AFLP, MSAP	Gao et al. (2010)
Gossypium hirsutum	Cotton	Callus culture	Chromosome count, RAPD	Jin et al. (2008)
Japonica rice	Rice	Callus culture	SSR Marker	Gao et al. (2011)
Lilium tsingtauense Gilg.	Martagon lily	Embryogenic culture	RAPD, microsatellite marker	Yang et al. (2010)
Musa acuminate	Desert banana	Genotype	RAPD	Sheidai et al. (2008)
Nothapodytes foetida (wight) sleumer	–	Embryogenic culture	Microsatellite marker	Chandrika et al. (2010)
Oryza sativa	Rice	Activation of transposable element	Microsatellite marker, transposon display	Gao et al. (2009)
Oryza sativa	Rice	Gamma rays	Morphology, PCR analysis	Joshi and Rao (2009)
Pinus pinaster	Maritime pine	Number of subcl.	Morphology	Marum et al. (2009)
Solanum	Potato	Genotype	Microsatellite marker	Aversano et al. (2009)
Solanum tuberosum	Potato	Embryogenic culture	Chromosomal count	Sharma et al. (2007)
Sorghum bicolor	Sorghum	Explants	Microsatellite marker	Zhang et al. (2010)
Theobroma cacao	Cocoa	Embryogenic culture	Cleaved amplified polymorphic sequence (CAPS)	Rodriguez Lopez et al. (2010)
Triticum aestivum	Wheat	Callus culture	Morphology	Arun et al. (2007)
Triticum aestivum	Wheat	Gamma rays	Lysozyme pattern	El-Sayed et al. (2007)
Vitis vinifera	Grapevine	Embryogenic culture	Chromosome count, microsatellite marker	Prado et al. (2010)
Zea mays	Maize	Callus culture	RAPD	Matheka et al. (2008)

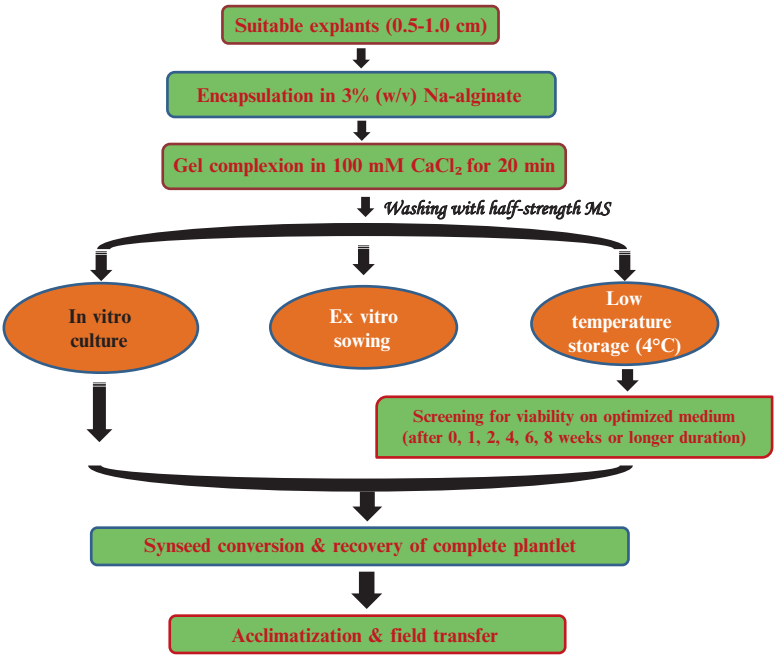
Suitable explants (0.5-1.0 cm)
Encapsulation in 3% (w/v) Na-alginate
Gel complexion in 100 mM $CaCl_2$ for 20 min
Washing with half-strength MS
In vitro culture
Ex vitro sowing
Low temperature storage (4°C)
Screening for viability on optimized medium (after 0, 1, 2, 4, 6, 8 weeks or longer duration)
Synseed conversion & recovery of complete plantlet
Acclimatization & field transfer

Fig. 2.1 Artificial seed production

somatic embryos, shoots, or other tissues which can be used for sowing under in vitro or ex vitro conditions". When the encapsulated explants evolve in complete plantlets (with shoot and root systems) in vitro or in vivo conditions, the product of the encapsulation is defined as synthetic seed or artificial seed or synseed (Sharma et al. 2013) (Figs. 2.1 and 2.2).

Production of artificial seeds has unravelled new vistas in in vitro plant biotechnology, such as large-scale clonal propagation, delivery of clonal plantlets, germplasm conservation, breeding of plants in which propagation through normal seeds is not possible, genetic uniformity and easy storage and transportation (Fig. 2.3). Artificial seed technology offers several potential advantages: (1) easier handling, (2) low production cost, (3) easier exchange of germplasm between different countries, (4) genetic uniformity of recovered plantlets, (5) direct delivery to the field, (6) to shorten the life cycle and (7) reduction of the storage space (Sharma et al. 2013).

2.10.1 Artificial Seed vs Natural Seed

Natural seed is a mature ovule in which food is stored in either endosperm or cotyledon. In other words, a natural seed is a zygotic embryo covered by several protective layers. Due to the presence of these protective layers, they are desiccation

Fig. 2.2 In vitro conversion of synthetic seeds into complete plantlets of *Spilanthes mauritiana* (**a**). Encapsulated nodal segments in 4 % Na-alginate with MS + 5.0 μM BA + 0.5 μM IAA inoculated on MS basal medium, 1-week-old culture (**b**). Conversion of abovesaid synseeds into complete plantlets (shoot and root development),4-week-old culture (**c**). An acclimatized plantlet of *S. mauritiana* in Soilrite

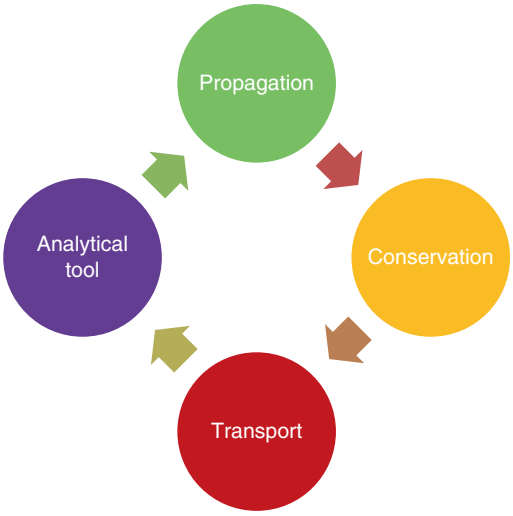

Fig. 2.3 Synthetic seed applications

tolerant. This property is used for germplasm preservation in seed repositories. Differently to zygotic embryos, the somatic embryoids could not germinate on its own; they are naked and need nutritional supply for physiological activities required for germination, growth and development into complete plantlets. The synthetic seeds are similar to the natural seeds because of the provision of artificial endosperm in the form of capsule which must contain all the required nutrients and hormones necessary for the germination and development of encapsulated propagules. They are formed after the encapsulation of somatic embryos having the artificial seed coat of gel and nutrients. Artificial seeds have great potential for large-scale production of plants at low cost as an alternative to true seeds (Roy and Mandal 2008).

2.10.2 Types

The following are the three types of artificial seeds produced till date:

1. *Desiccated artificial seed*: Coated desiccated embryos represent an ideal form of synseed (Pond and Cameron 2003) for which somatic embryos are first hardened to withstand desiccation before encapsulation. Such types of synseeds can only be produced in those plants whose somatic embryos are desiccation-tolerant (*see* Sharma et al. 2013). Kitto and Janick (1985) for the first time produced desiccated artificial seeds by encapsulating carrot somatic embryos in a chemical readily soluble in water, polyoxyethylene, which form a thin film and embryoids after drying. Similarly, Janick et al. (1989) developed desiccated artificial seeds of carrot by using polyoxyethylene glycol to coat the mixture of somatic embryo and calli.
2. *Hydrated artificial seed*: Hydrated artificial seeds are produced in such cases where the embryoids are sensitive to desiccation. The most used method to produce artificial seeds is inotropic gelation of sodium alginate by calcium ions. Redenbaugh et al. (1984) developed the technique of hydrogel encapsulation of individual embryoids of alfalfa. Since then hydrogel encapsulation remains to be the most practiced technique of artificial seed production.

2.10.3 Preparation of Synthetic Seeds

1. *Encapsulating agents*: The large number of encapsulating agents has been tested in time for their capacity to produce beads, such as agar, agarose, alginate, carboxymethyl cellulose, carrageenan, ethyl cellulose, gelrite, guar gum, nitrocellulose, polyacrylamide, polyox and sodium pectate (Datta et al. 2001, Saiprasad 2001). For instance, Kitto and Janick (1985) screened eight chemical compounds for the encapsulation of propagules including cells, cell aggregates, callus

clumps, etc. and found that a water-soluble resin, the polyethylene oxide homopolymer ("polyox"), was a more appropriate material to encapsulate embryogenic suspension of carrot. Redenbaugh et al. (1988), after thorough screening of coating material for synthetic seed production, recommended the use of Na-alginate solution coating, containing propagules to be dipped in calcium chloride solution for the formation of hardened Ca-alginate gel by an ion-exchange reaction. Somatic embryos of alfalfa and celery were the first to be alginate-coated, reaching a synseed germination rate (=conversion to plantlets) which in alfalfa was over 85 %. Using this chemical combination, they were able to encapsulate embryoids of alfalfa and closely with germination success rate (= conversion to plantlets) of over 85 % (in alfalfa). Since then, the alginate became by far the most used gelling agent for synthetic seed preparation in different plant species (Bapat et al. 1993; Sharma et al. 2009a, b; Sharma and Shahzad 2011; Sharma et al. 2014; Parveen and Shahzad 2014; Sharma and Shahzad 2014; Sharma et al. 2015; Shaheen and Shahzad 2015).

2. *Components of the artificial matrix*: The synthetic endosperm or artificial matrix is composed of minerals and vitamins of the MS medium or other suitable culture medium supplemented with different plant growth regulators (PGRs) like indoleacetic acid (IAA), α-naphthalene acetic acid (NAA), 6-benzyl aminopurine (BA), kinetin (Kn), 2-isopentyl adenine (2-iP) and sucrose. Depending on the encapsulation treatment applied, agar is replaced by sodium alginate (2, 3 and 4 %).
3. *Encapsulation procedure*: The following are different strategies used for artificial seed production in various plant species:

   (a) *Drop-by-drop method*: In this method, isolated somatic embryos or non-embryogenic propagules are submerged in sodium alginate solution (1–4 % w/v depending on the plant species) and subsequently suctioned through a micropipette to provide a protective capsule. In order to seal the capsules, they are then submerged in a complexing solution of $CaCl_2 \cdot 2H_2O$ or $CaCl_2$ for a determined period of time (15–20 min) followed by washing in sterile water for 40 min then transferred to sterilized filter paper placed in Petri dishes for 5 min. This process is carried out under aseptic conditions in a laminar flow chamber. Finally, the artificial seeds are cultivated on nutrient medium in Petri dishes with macro- and micronutrients supplemented with sucrose and agar-agar. These artificial seeds can also be used for storage purpose or directly sown in suitable planting substrate under ex vitro conditions.

   (b) *Partially encapsulated synseed*: In this technique, Ca-alginate beads (without propagules) are produced, and then by making a hole in the bead, somatic embryo is inserted. In this approach, the embryo primordium remains exposed which in turn prevents any possible physical inhibition of embryoid conversion into plantlets, as complete encapsulation. Sometime showed resemblance to the emergence of radical/plumules (i.e. enable to break coating). This method was proposed by Mamiya and Sakamoto (2001).

An advantage of this method is related to the fact that the embryo primordium is not embedded totally in the alginate matrix, thus preventing any possible physical inhibition to the embryo conversion to plantlet. However, the complexity of the technique, in comparison with the conventional "drop-by-drop" method, is a weak point which has prevented its mass acceptability.

(c) *Hollow beads*: Another encapsulation procedure was developed by Patel et al. (2000) with the aim of producing Ca-alginate hollow beads. In this method propagules of potato and carrot were suspended in calcium chloride and carboxymethyl cellulose solution followed by drying in Na-alginate solution.

(d) *Double-layered artificial seed*: Double-layered synseeds were prepared to overcome the problems caused by the use of sodium alginate such as loss of the nutritive substances and/or dehydration risk during conservation and transport. Micheli et al. (2002) developed double coat encapsulation and encapsulation-coating procedure in M.26 apple rootstock. They used a large quality of sucrose in inner layer of synseeds. To prevent the diffusion of sucrose from artificial seeds to non-sterilized substrate, artificial seeds were enveloped in a dialysis membrane. The enveloped artificial seeds germinated quickly in non-sterilized vermiculite.

2.11 Conservation of Germplasm

2.11.1 Methods of Cryopreservation of Germplasm and Plantlet Production

Cryopreservation is the preservation of plant genetic resources at ultralow temperature (−196 °C) which enables plant genetic resources to be conserved safely and cost-effectively. Successful cryopreservation technique requires that (1) there should be no intracellular freezing, (2) vitrification state of plant cells should be induced during cooling in liquid nitrogen (LN) and (3) the cryopreservation method should also be very simple for everyone to use effortlessly. During the last few decades, cryopreservation techniques have been applied for different plant organs, tissues and cells, and as a consequence, a variety of cryopreservation methods have been developed such as slow prefreezing method, vitrification method and dehydration method. With the invent of these techniques, now it is possible to preserve the tissues of some tropical plants in LN which were earlier thought to be not cryopreserved (Bajaj 1995; Towill and Bajaj 2002).

2.11.1.1 Slow Programmed Freezing or Prefreezing

Till 1980, slow programmed freezing was a major cryopreservation method for plant genetic resources. In this method, cells and tissues were packed in cryotube, and cryoprotectants such as dimethyl sulfoxide (DMSO), ethylene glycol (EG) and glucose were added. These cryoprotectants were used singly in most of the cases, but Finkle and Ulrich (1979) found that the regrowth percentage of the sugarcane germplasm was very high after cryopreservation when mixing of cryoprotectants was done. Using a programmable freezer or ethanol baths, the packed specimens were gradually cooled from −20 to −100 °C. Processing is performed near −7 to −8 °C in the middle of the freezing, which freezes cryoprotectant in a tube artificially. Intracellular moisture arrives at the surface of the ice through penetrating the plasma membrane and freezes; this is called "extracellular freezing". After freezing of specimens to a predetermined freezing temperature (−40 °C), they are immersed in LN. Cryopreserved tubes are warmed for 1–2 min using hot water (40 °C), and cryoprotectants are removed from a tube. After rewarming, samples are moved from the cryotube and recultured. In this method the cooling rate is important and it differs from 0.5 to 50 °C/min with plant species depending on the size of the plant germplasm. However, in case of the freezing speed of 2 °C/min or more, the regrowth after preservation tends to drop (Sugawara and Sakai 1974; Uemura and Sakai 1980). There are some plant tissues which freeze to death partially and in some cases the decrease in subsequent viability also exists (Grout and Henshaw 1980; Haskins and Kartha 1980).

2.11.1.2 Slow Unprogrammed Freezing or Simple Freezing

The advantage of this cryopreservation is that the plant samples can be preserved without a special programmable freezer, compared with slow programmed freezing. Plant tissues are added to the tube containing cryoprotectants, and the tubes are treated at room temperature (25 °C) for about 10 min and then kept at −30 °C for 30–120 min. After that, the tubes are immersed in LN. Cryopreserved tubes are warmed for 1–2 min using hot water (40 °C), and cryoprotectants are removed from the tube. After rewarming, samples are moved from the cryotube and recultured. Mixtures of glycerol and sucrose or DMSO and sorbitol are used as cryoprotectants in this method (Sakai et al. 1991; Niino et al. 1992; Maruyama et al. 2000). Although "naked" samples are used in this cryopreservation method, Kobayashi et al. (2005) used cells encapsulated with alginate beads in the suspension cells of tobacco.

2.11.1.3 Vitrification

This method has been the major cryopreservation method since Uragami et al. (1989) developed it using *Asparagus* culture cells. For the osmoprotection, plant tissues are added to the tube containing the loading solution (LS), and the beads in

Table 2.5 Components of major plant vitrification solutions referred from previous reports

Component(G/L)	PVS1	PVS2	PVS3
Glycerol	220.0	300.0	500.0
Ethylene glycol	150.0	150.0	–
Propylene glycol	150.0	–	–
Dimethyl sulfoxide (DMSO)	70.0	150.0	–
Sucrose	–	136.9	500.0
Sorbitol	91.1	–	–

Uragami et al. (1989) and Sakai et al. (1990)

tubes are osmoprotected for about 30 min at room temperature. After loading, LS is removed from a tube, and a new vitrification solution is added for the dehydration of plant tissues. In most of the cases, dehydration is done at 0 °C using the vitrification solution. Plant vitrification solution 2 (PVS2; Sakai et al. 1990) is utilized in maximum of the cases. However, many other vitrification solutions are also used (Table 2.5). Plant tissues are immersed in LN, and the cryopreserved tubes are warmed for about 1–2 min using hot water (40 °C). After that the vitrification solution is removed from the tube. Then unloading solution (the liquid medium supplemented with 1.2 mol/L sucrose) is added to a tube, and cryoprotectants are removed from plant tissues for 30 min at 25 °C. In many cases, the above-mentioned liquid media (LS, PVS and unloading solution) were adjusted at pH 5.7–5.8, but without plant growth regulators. After unloading, samples are removed from the cryotube and recultured (Table 2.5).

2.11.1.4 Encapsulation–Vitrification

The encapsulation–vitrification method was first reported by Matsumoto et al. (1995) using shoot apices of *Wasabia japonica*. The advantage of this method is that regrowth of plant germplasm after cryopreservation increased significantly by encapsulating plant samples with alginate beads. The encapsulation of plant germplasms prevents the samples from damage during vitrification procedures. Conversely, due to encapsulation–dehydration, treatment time becomes long compared with that of vitrification, but cryopreservation becomes complicated. Plant tissues are dipped in calcium-free liquid medium supplemented with 0.4 mol/L sucrose, 30.0 g/L sodium alginate and glycerol (1.0–2.0 mol/L). This mixture including a plant cell or tissue was then added drop by drop to the liquid medium containing 0.1 mol/L calcium chloride, forming beads of about 5 mm in diameter. Encapsulated specimens are kept in culture bottles containing LS for osmoprotection for 16 h at room temperature (25 °C). LS is a liquid culture medium containing sucrose (0.75–0.8 mol/L) and glycerol (2.0 mol/L). After loading, LS is removed from the bottle, and PVS is added for the dehydration of plant tissues. Similar to vitrification, the dehydration using PVS is performed at 0 °C in light. After dehydration of PVS, encapsulated samples are moved to a cryotube containing fresh PVS

and dipped in LN. Cryopreserved tubes are warmed using hot water for 1–2 min, and the vitrification solution is removed from the tube. After that unloading solution is added to the tube and cryoprotectants are removed from plant tissues for 30 min at 25 °C. After unloading, samples are moved from the cryotube and recultured.

2.11.1.5 Simplified Encapsulation–Vitrification

This method was first reported by Hirai and Sakai (2002) using shoot apices of sweet potato (*Ipomoea batatas*). The operating procedure in this method is the same as encapsulation–vitrification. The composition of LS is, however, different. LS of simplified encapsulation–vitrification contains high concentration of glycerol (2.0 mol/L) and sucrose (1.6 mol/L), and the viscosity of LS is high. Although, this method is successful with sweet potato, there are some other plant species which cannot be cryopreserved using high concentration of glycerol (Hirai and Sakai 1999).

2.11.1.6 Droplet Method

The droplet method was first reported by Schäfer-Menuhr et al. (1994, 1997) using potato (*Solanum tuberosum*) apices. The operating procedure is the same for vitrification. However, the LS immersion protocol differs compared with that in the vitrification method. After treatments by LS and PVS, plant samples are kept on aluminium foil which is sterilized and cut into small pieces. One drop of PVS is put onto plant samples and the whole aluminium foil is dipped in LN. The aluminium foil after cryopreservation is taken out from LN, and one drop of unloading solution supplemented with 1.0 mol/L sucrose is dripped onto the freezing samples. After rewarming, samples are moved from the cryotube and recultured. Wesley-Smith et al. (2001) used slush nitrogen (−210 °C) and an isopentane (−160 °C) instead of liquid nitrogen in order to cool a plant sample quickly. Furthermore, the droplet method can reportedly obtain a high regrowth percentage after cryopreservation in tropical plants which are difficult to cryopreserve (Pennycooke and Towill 2000, 2001; Leunufna and Keller 2003; Panis et al. 2005).

2.11.1.7 Dehydration

Dehydration was first reported by Uragami et al. (1990) using *Asparagus* lateral buds. This method is superior to vitrification, because it does not need PVS. However, in dehydration there is influence of humidity on drying by airflow, and dried samples are easily crushed with tweezers. Plant tissues are kept on the sterilized filter paper or nylon mesh and cut into small pieces. Samples are dehydrated by silica gel (Uragami et al. 1990) or airflow (Shimonishi et al. 1991; Kuranuki and Yoshida 1996) before immersion in LN. It is described that the optimal moisture of the

sample is 10–30 % for survival after cryopreservation in the dehydration method (Uragami et al. 1990; Shimonishi et al. 1991; Kuranuki and Yoshida 1996). After the dehydration, germplasms are moved to a cryotube and dipped in LN. Cryopreserved tubes are warmed at room temperature or using hot water (40 °C) for 1–2 min. After rewarming, samples are removed from the cryotube and recultured.

2.11.1.8 Encapsulation–Dehydration

The encapsulation–dehydration method was first reported by Fabre and Dereuddre (1990) using shoot apices of potato. This method surpasses dehydration in that the regrowth of plant germplasm after cryopreservation is markedly increased by encapsulating plant samples with alginate beads. Furthermore, encapsulated samples are difficult to be crushed with tweezers compared with the dehydration method. Plant tissues are dipped in a calcium-free liquid medium supplemented with 0.4 mol/L sucrose and 30.0 g/L sodium alginate. The mixture was added drop by drop to the liquid medium containing 0.1 mol/L calcium chloride, forming beads of about 5 mm in diameter. The above-mentioned liquid media (30.0 g/L sodium alginate and 0.1 mol/L calcium chloride) were adjusted at pH 5.7–5.8 but without plant growth regulators. Encapsulated germplasms are added to the culture bottle containing LS for the osmoprotection. Beads in the bottles are osmoprotected for 16 h at room temperature. LS is the liquid culture medium containing sucrose (0.75–0.8 mol/L). After loading, LS is removed from the bottle. Loaded samples are put on sterilized filter papers and dehydrated by silica gel for 3–7 h before immersion in LN. After dehydration by silica gel, encapsulated samples are moved to a cryotube and immersed in LN. Cryopreserved tubes are warmed using hot water for 1–2 min. After rewarming, samples are moved from the cryotube and recultured. In encapsulation–dehydration, the addition of glycerol besides sucrose in LS reportedly enhances the regrowth percentage of cryopreserved samples. The optimal concentration of glycerol in LS is 0.5–2.0 mol/L for regrowth of cryopreserved specimens (Matsumoto and Sakai 1995; Kami et al. 2005, 2008).

2.11.1.9 Newly Developed Encapsulation–Dehydration

This newly developed encapsulation–dehydration method was first reported by Sakai et al. (2000). The operating procedure is the same as used in encapsulation–dehydration. However, the LS composition differs. LS of the newly developed encapsulation–dehydration includes a high concentration (2.0 mol/L) of glycerol besides sucrose. Therefore, the loading time of this method (1 h) is shorter than that of encapsulation–dehydration (16 h).

The storage of vegetative material, cell cultures, seeds, spores and DNA at ultralow temperature (−196 °C) provides a safe and cost-effective method for the long-term storage of genetic resources. The importance of cryopreservation as a conservation tool has been reported by many authors (Stacey et al. 1999; Engelmann

and Dussert 2000; Engelmann 2004), and it is currently the only available technique for the safe long-term storage of species with recalcitrant seeds or those that are vegetatively propagated (Engelmann and Dussert 2000). Cryopreservation can be employed for a wide range of vegetative material such as shoot tips, axillary buds, embryonic axes, bryophyte and fern, somatic embryos, callus and suspension cultures. These potential explants allow many possible applications for this technique. Indeed, cryopreservation is being used for the routine banking of germplasm in some taxa (Stacey et al. 1999). Two key advances in methodology have made this possible: the development of vitrification using PVS2 solution (Sakai et al. 1990) and the encapsulation–dehydration technique (Fabre and Dereuddre 1990). Plant vitrification solution 2 (PVS2) is a highly concentrated cryoprotective solution supplemented with glycerol, ethylene glycol and DMSO. This solution allows cells to dehydrate sufficiently, permitting vitrification during freezing and subsequent steps in cryopreservation. Encapsulation–dehydration involves encapsulation of plant sample in a protective matrix and its subsequent controlled dehydration. Advantages of encapsulation include easier manipulation of delicate tissues and direct protection during dehydration and thawing processes (Paul et al. 2000). It is necessary to consider the ecological origin and nature of each species to be treated (e.g. whether it is desiccation and/or freezing tolerant and the type, size, condition and culture of the explant) to identify potentially useful cryopreservation methods (Turner et al. 2000; Baek et al. 2003; Towill et al. 2004). Preculture with abscisic acid (Pence 2000; Bian et al. 2002; Burch and Wilkinson 2002), cytokinins (Turner et al. 2000, 2001a), specific sugars and sugar alcohols (Suzuki et al. 1998; Turner et al. 2001b; Schulte and Reski 2004) reduced levels of NH_4^+ and NO_3^- (Decruse et al. 2004); low levels of DMSO (Decruse et al. 1999; Sharma and Sharma 2003; Schulte and Reski 2004) and low temperature (Sharma and Sharma 2003) have been shown to improve dehydration tolerance, cryoprotection or recovery of plants of threatened plant species. The use of 0.75 M sucrose during the freezing protocol is well documented and has been used successfully in a number of species (González-Benito and Pérez 1997; Decruse et al. 1999; Pence 2001). Preculture of the endangered Australian plants, *Anigozanthos* and *Conostylis*, with high levels of glycerol significantly improved survival rates (Turner et al. 2001b). Additional research on *Anigozanthos viridis* revealed that the polyols, glycerol, sorbitol and inositol resulted in higher survival rates than the sugars, viz. sucrose, glucose, trehalose and raffinose, when used at the same osmolarity (0.4 M) or at the equivalent concentration of total hydroxyl groups (Turner et al. 2001a). While the use of PVS2 provides a good method for vitrification, one of its primary components, DMSO, can be toxic. Wilkinson et al. (1998) presented an interesting modification to the encapsulation–dehydration method, where shoot tips of the endangered species *Cosmos atrosanguineus* were encapsulated on alginate-covered paper strips to allow easier transfer of the samples and permit better positioning of the explant. This method has been used successfully for the cryopreservation of protonemata from the endangered bryophyte, *Ditrichum cornubicum* (Burch and Wilkinson 2002), and is currently being used for the cryopreservation of a wide range of bryophyte species at RBG, Kew, UK. Traditionally, samples are frozen at a controlled rate or plunged directly

into liquid nitrogen. They are recovered normally by thawing either at ambient temperature or at 40 °C. Leunufna and Keller (2003) presented an alternative method in which they used the droplet method to cryopreserve apical buds. They found that freezing apical meristems in droplets of cryoprotective solution or PVS2 on small aluminium foil pieces increased survival and regrowth in comparison to the normal vitrification protocol. This success was, in part, attributed to more rapid and uniform heat distribution in the small drops. Dumet et al. (2002) thawed encapsulated *Pelargonium* meristems by dropping them directly into 0.75 M sucrose-enriched liquid medium at 20 °C for 1 min, yielding significantly higher survival rates than thawing at 40 °C or at ambient temperature. Success was attributed to a reduced thawing rate and possible additional osmoprotection during the process.

2.11.2 Slow Growth Method of Tissue Culture

Micropropagation has the potential for development as a routine method for the in vitro conservation of rare/endangered/threatened plant species. Long-term storage of material in culture is challenging, and the potential applications of cryopreservation are significant in this area. The minimal growth storage methods are usually appropriate to a wide range of fruit trees to extend the ordinary subculture duration for a few weeks to 12 months for future needs and to preserve germplasm as in vitro gene bank in limited spaces.

Slow growth storage technique is commercially used in laboratories to maintain stock cultures through low temperatures. To achieve this purpose, various methods have been used such as by reducing concentration of basal medium (Moriguchi and Yamaki 1988), removal of cytokinins and addition of growth retardants (Gunning and Lagerstedt 1985) and adding mannitol as osmoticum (Wanas et al. 1986) at normal temperature. However, the most reliable and simple method for growth retardation is lowering the culture temperature. Attempts have been made in several laboratories to conserve *Pyrus* germplasm by using this technique (Wanas et al. 1986; Wilkins et al. 1988; Moriguchi et al. 1990; Moriguchi 1995). Depending on the plant species, these stored plants can be micropropagated rapidly when desired. Medium-term (3 months to 3 years) storage conditions for in vitro cultures of temperate genera are typically 4 or 5 °C in darkness (Druart 1985, Marino et al. 1985; Wilkins et al. 1988; Reed 1992). Lundergan and Janick (1979) described the storage of shoots of apple scion variety (Golden Delicious) at reduced temperature (1 and 4 °C) for 1 year. Barlass and Skene (1983) have reported successful storage of seven species of *Vitis* (grapes) for periods up to 12 months at 9 °C. Marino et al. (1985) have reported successful storage of shoots of three *Prunus* rootstock genotypes for several months at reduced temperature. The shoots of peach and cherry could be preserved for 120 days at 4 °C. Twenty pear (*Pyrus*) cultivars of seven species cultured on MS basal nutrient medium and stored in vitro culture methods like meristem cryopreservation as long-term and medium-term storage techniques involving low temperatures (1–4 °C) for germplasm preservation programmes (Bell and Reed 2002).

The principle of reduced growth storage depends on the manipulation of culture condition to allow the culture to remain viable at very slow growth rate. In this technique, the growth of explants is controlled by adding growth-retarding agents in the medium to allow the culture to remain viable but with almost negligible growth rate, which could be targeted for *ex situ* conservation of plants (Mandal 1997). Micropropagation and slow/reduced growth method are the two commonly used in vitro conservation methods. Micropropagation is the powerful tool used to conserve elite, rare and endangered species. It is an effective means for rapid multiplication of species and *ex situ* conservation. Therefore, rapid and large-scale production of medicinal and aromatic plants employing micropropagation has gained significant interest (Asamenew and Narayanaswamy 2004). The main drawback of plant cell culture is the danger of spontaneous changes. Therefore, continuous culture of plant cells is often desirable, and in this situation, slow growth technique will be a potential tool for a more efficient and cost-effective rapid propagation system for medium-term storage (Dube et al. 2011). Plant tissue cultures can be stored for the medium term by maintaining in a slow growth state (Negri et al. 2000; Mandal et al. 2000). Iriondo and Perez (1996) stored shoots of an endangered plant *Centaurium rigualii* at 58 °C with a 16 h photoperiod and achieved a survival rate of 90 % after 3 years. The orchid *Ipsea malabarica* was maintained successfully in a state of slow growth for 20 months on half-strength MS medium without the addition of sucrose or PGRs by Martin and Pradeep (2003). Production and storage of synthetic seeds via the encapsulation of somatic embryos and other propagules is another alternative (Standardi and Piccioni 1998). Mandal et al. (2000) prepared synthetic seeds of different species of basil (*Ocimum* spp.) from axillary buds. Encapsulated protocorms of orchids, *Cymbidium longifolium, Agrostophyllum myrianthum*, *Phaius tankervillae* and *Renanthera imschootiana*, were stored at 48 °C and showed 70 % viability after more than 6 months (Devi et al. 1998) (Table 2.6).

Table 2.6 List of some important plant species conserved through slow growth method

Plant	Explant stored	References
Epidendrum chlorocorymbos	Three-month-old in vitro-raised seedlings	Guadalupe Lopez-Puc (2013)
Glycyrrhiza glabra	Shoot apices	Srivastava et al. (2013)
Phoenix dactylifera	Shoot tips	El-Dawayati et al. 2013
Pyrus spp.	Shoot tips	Ahmed and Anjum (2010)
Coleus forskohlii	Nodal segments	Dube et al. (2011)
Piper aduncum and *Piper hispidinervum*	In vitro shoots with one lateral bud	Loureiro da Silva and Scherwinski-Pereira (2011)
Elettaria cardamomum	Shoot tips	Tyagi et al. (2009)
Allium sativum	Bulbs	Hassan et al. (2007)
Vanilla spp.	Shoot tips and nodal segments	Divakaran et al. (2006)
Cocos nucifera	Embryos	Sukendah and Cedo (2005)
Solanum tuberosum	Microplants	Sarkar et al. (2001)

2.12 Summary and Future Prospects (Rewrite)

Plant tissue culture technology has become an integral part of playing an increasingly important role in basic and applied studies, including crop improvement. In modern agriculture, only about 150 plant species are extensively cultivated, and crop improvement has reached the limits for their improvement by traditional methods. Advances in plant tissue culture technology have made it integral part of basic and applied studies. Furthermore, combining one or more techniques of plant cell culture can be an effective tool for the production of high-value recombinant proteins at large-scale crop improvement, incorporation of desired genetic trait, production of transgenics and conservation of germplasm. The ability to accelerate the conventional multiplication rate can be of great benefit to many countries where a disease or some climatic disaster wipes out crops. Slow growth in vitro storage and cryopreservation are being proposed as solutions to the problems inherent in field gene banks. Thus, the future generations will be able to have access to genetic resources for simple conventional breeding programmes or for the more complex genetic transformation work as well. New opportunities have been created for producers, farmers and nursery owners for high-quality planting materials of fruits, ornamentals, forest tree species and vegetables where plant production can be carried out throughout the year irrespective of season and weather.

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Chapter 3
Plant Genetic Resources: Their Conservation and Utility for Plant Improvement

Tapan Kumar Mondal and Krishna Kumar Gagopadhyay

Abstract Biological diversity denotes the variability among the living organisms. Biodiversity provides the basic biotic resources that sustain the human race. This includes diversity within species and between species of ecosystems. Biodiversity is not merely a natural resource; it is an embodiment of cultural diversity and the diverse knowledge of different communities across the world. India is also a vast repository of traditional knowledge associated with biological resources. Currently, there has been growing realization that biodiversity is the basis of sustainable agricultural production and food security as well as important factor for environment conservation. In India, agro-biodiversity deserves special attention to ensure conservation of valuable germplasm for perpetuity, sustainable use and development, and livelihood security. Thus, a better understanding of genetic diversity and its distribution is essential for its efficient conservation and use. In this chapter, we review the genetic diversity in plant genetic resources in India along with the methods of their management for plant improvement.

3.1 Indian Plant Biodiversity

India, with a total area of 329 mha (2.4 % of world's land area), is the seventh largest country in the world. The diverse ecology together with unique geological and cultural features has resulted to a wide diversity of habitats and sustains immense plant diversity at all levels. Out of 1.75 million species recorded so far in the world, India accounts for 7–8 % of recorded species of the world and ranks seventh in mammals, ninth in birds, and fifth in reptiles. In terms of endemism of vertebrate groups, India's position is tenth in birds with 69 species, fifth in reptiles with 156 species, seventh in amphibians with 110 species, and tenth in plants with 5000 species.

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Table 3.1 Recorded plant species on earth[a]

Taxonomic group	Number of species		% of world flora
	World	India	
Angiosperms	25,0000	17,500	7.0
Gymnosperms	65,048	48	7.4
Pteridophytes	10,000	1200	12
Bryophytes	14,500	2850	19.7
Lichens	13,500	2075	15
Fungi	70,000	14,500	20.7
Algae	40,000	6500	16.3
Virus/bacteria	8050	850	10.6
Total	*4,06,700*	*45,523*	*11.8*

[a]Anonymous (2012)

India with 45500 different plant species represents nearly 11 % of the world's known plant diversity. In higher plants (angiosperms), the country has about 17,527 different flowering plants accounting for more than 7 % of the world's known flowering plants, out of which ~35 % of flowering plants are endemic (Table 3.1).

The varied topographic, edaphic, and climatic conditions have yielded various types of ecosystems and habitats such as forests, grasslands, wetlands, coastal as well as marine ecosystems, and deserts. While the mountainous region occupied close to 100 mha, arid and semiarid zones are over 30 mha, and the coastline is about 8000 km long in India.

3.1.1 Species and Ecosystem Diversity

Species diversity means variety of species, i.e., number as well as kinds of living organism available within a region. The ecosystem diversity indicates various types of habitats, communities, and ecological processes that are found in the biosphere. It is not easy to measure since the boundary of the communities that constitute the different sub-ecosystems is elusive. According to biogeographic classification, India represents (1) two "realms," the Himalayan region represented by Palearctic realm and the remaining of the subcontinent represented by Malayan Realm; (2) five "biomes," tropical humid forests, tropical dry deciduous forests (including monsoon forests), warm deserts, and semideserts, coniferous forests, and Alpine meadows; and (3) ten biogeographic zones and 27 biogeographic provinces (Table 3.2).

Agro-biodiversity describes the variability of economically useful organisms such as animals, plants, and microorganisms that are used directly or indirectly for livelihood of the people. It comprises the diversity of genetic resources (varieties, breeds) which are used for food, fiber, fodder, pharmaceuticals, and fuel. It also includes the variability of non-harvested but beneficial organism that is required to increase either our yield (soil microorganisms, predators, and pollinators) or those

Table 3.2 Different biogeographic zones of India[a]

S. no.	Zones	Provinces	Geographical area of India (%)
1.	Trans-Himalaya	1A: Himalaya-Ladakh mountains	3.3
		1B: Himalaya-Tibetan Plateau	2.2
		1C: Trans-Himalaya Sikkim	<0.1
2.	The Himalaya	2A: Himalaya-Northwest Himalaya	2.1
		2B: Himalaya-West Himalaya	1.6
		2C: Himalaya-Central Himalaya	0.2
		2D: Himalaya-East Himalaya	2.5
3.	The Indian Desert	3A: Desert, Thar	5.4
		3B: Desert, Kachchh	1.1
4.	The Semiarid	4A: Semiarid, Punjab Plains	3.7
		4B: Semiarid, Gujarat Rajputana	12.9
5.	The Western Ghats	5A: Western Ghats-Malabar Plains	2.0
		5B: Western Ghats-Western Ghats mountains	2.0
6.	The Deccan Peninsula	6A: Deccan Peninsular-Central Highlands	7.3
		6B: Deccan Peninsular-Chota Nagpur	5.4
		6C: Deccan Peninsular-Eastern Highlands	6.3
		6D: Deccan Peninsular-Central Plateau	12.5
		6E: Deccan Peninsular-Deccan South	10.4
7.	The Gangetic Plains	7A: Gangetic Plain-Upper Gangetic Plains	6.3
		7B: Gangetic Plain-Lower Gangetic Plains	4.5
8.	The coasts	8A: Coasts-West coast	0.6
		8B: Coasts-East coast	1.9
		8C: Coasts-Lakshadweep	<0.1
9.	Northeast India	9A: Northeast-Brahmaputra Valley	2.0
		9B: Northeast-Northeast hills	3.2
10.	Islands	10A: Islands-Andaman	0.2
		10B: Islands-Nicob	0.1

[a]Anonymous (2007)

which support our agroecosystems (FAO 1999). It is an essential active component of biological diversity and basic to the farming systems which includes the wide variety of species and means by which farmers can harness biological diversity in a sustainable manner for their benefit. It encompasses many types of biological resources related to agriculture, livestock, fish species, and useful microbes, along with their wild species.

3.1.2 Genetic Diversity

It refers to the variations at genome level within a population of species. It constitutes genetic variations within population or varieties within a species or distinct population of the same species. The extent of diversity of genomic level depends upon several factors such as size of the population, reproductive biology, and migration, which often are not visible, if the variation is too wide. The Indian gene center is among the 12 mega-diversity regions of the world and one of the eight Vavilovian centers of origin and diversity of crop plants with 166 crop species of Hindustani center origin with more than 583 cultivated species, 334 wild species, and close relatives of cultivated plants. Around 1500 wild edible plant species (145 species of roots and tubers, 521 of leafy vegetables, 101 of buds and flowers, 647 of fruits, and 118 of seeds and nuts) are widely exploited by native tribes. Additionally around 9500 useful plant species have been reported from the country, of which around 7500 are with medicinal value and 3900 are multipurpose including edible species. Canonically, variation at the genomic level can be detected by DNA-based molecular markers which are utilized for effective *ex situ* conservation of plant genetic resources (PGRs), aimed to resolve various biological questions, associated to the PGR management (Bansal et al. 2014). There are several molecular markers available today, and a detailed account of which is beyond the scope of this article. Nevertheless, on the basis of technical methodology, it can be divided broadly in two different categories, i.e., "PCR based" and "non-PCR based." RAPD, ISSR, SSR, and MAAP are examples of former groups, whereas AFLP, RFLP, and VNTR are the examples of later group (Mondal and Sutoh, 2013; Ganie and Mondal 2015). However, the basic principle is to utilize various types and position of variation in the genome that can make the molecular identity of the individual. Mainly molecular markers are used (1) to determine whether a genotype is catalogued correctly and maintained properly and whether genetic erosion has occurred in an accession or population over time; (2) to determine the degree of similarity among the genotypes within a collection; (3) the partitioning of variation among the genotypes, accessions, populations, and species; and (4) to detect a particular gene or nucleotide sequence in a genotype, gene bank accession, in situ population, chromosome, or cloned DNA segment (Karp et al. 1997).

3.2 Conservation of Biodiversity

The prerequisite for sustainable utilization of biodiversity of any kinds is the conservation of the same. The conservation involves two basic approaches, i.e., *ex situ* and in situ conservation, which are complementary by nature and described below:

3.2.1 In Situ Conservation

Conserving the genetic resources and protections of their ecosystems or natural habitats is known as in situ conservation. This approach allows fitness of species and continuous evolutionary development under natural selection, but species remain vulnerable to the unexpected natural calamities. There are advantages in in situ conservation (IUCN-UNESCO/FAO 1989) such as it usually conserves a large range of alleles including rare allele. Additionally, it is particularly helpful to species that cannot be preserved in outside the natural habitats. Again, they are of three types: (1) species of complex ecosystem, e.g., moist tropical forests, where the species cannot survive as a monoculture due to their dependence on other species; (2) species with dormant seeds or seeds with fugacious germination where dormancy cannot be removed by conventional methods; and (3) species with highly specialized breeding system. For example, if the breeding is dependent on a single type of bird, insect, or bat as pollinator, then those species have to depend on a particular ecosystem as per the choice of the pollinators. Besides, it helps natural evolution to occur, a valuable option for disease- and pest-resistant species, which evolve together with their parasites. It also facilitates research on species at their natural habitats and ensures protection of associated species. The established natural habitats are two types:

1. Biosphere reserves (BRs): BRs are special environments for both human being and the nature and are living example of how man and nature can coexist by respecting each other's needs. Fifteen biosphere reserves have been designated and ten potential areas are proposed in India (Table 3.3). In the case of biosphere reserve, the conservation of species is done through ecosystem.
2. Protected areas: It is conservation through habitat approach, which targets management of target species through impressive protected areas network, India's major strength. There are currently 661 protected areas, out of which 515 wildlife sanctuaries, 99 national parks, 43 conservation reserves, and 4 community reserves.

3.2.2 Ex Situ Conservation

Preserving or protecting the genetic resources away from the natural habitat has several advantages. The different methods are:

1. Seed gene bank: Conservation of orthodox seeds at subfreezing temperature (-20 °C).
2. Cryo bank: Storage of orthodox, intermediate, and recalcitrant (embryonic axes) in vapor/liquid nitrogen (-193 °C).
3. In vitro tissue culture: Conservation of cells or tissue under laboratory condition with a slow growth of cultures under a range of temperature (4–25 °C).

Table 3.3 Indian biosphere reserves[a]

S. no.	Biosphere reserves	Representative states
1.	Nilgiri[b]	Karnataka, Tamil Nadu, and Kerala
2.	Agasthyamalai	Tamil Nadu and Kerala
3.	Gulf of Mannar[b]	Tamil Nadu
4.	Seshachalam	Andhra Pradesh
5.	Pachmarhi	Madhya Pradesh
6.	Panna	Madhya Pradesh
7.	Achanakmar Amarkantak	Madhya Pradesh and Chhattisgarh
8.	Kachchh	Gujarat
9.	Nanda Devi[b]	Uttarakhand
10.	Cold Desert	Himachal Pradesh
11.	Manas	Assam
12.	Dibru-Saikhowa	Assam
13.	Dihang-Dibang	Arunachal Pradesh
14.	Nokrek	Meghalaya
15.	Khangchendzonga	Sikkim
16.	Sunderban[b]	West Bengal
17.	Simlipal	Orissa
18.	Great Nicobar	Andaman and Nicobar Islands

[a]Anonymous (2007)
[b]Recognized by UNESCO on World Network of Biosphere Reserves

4. Field repositories or field gene bank: Most organizations dealing with perennial or vegetatively propagated domesticated crops mainly horticultural crops, forestry species, and medicinal and aromatic plants have field repositories for conservation of plant genetic resources.
5. Botanical garden: There are more than 200 publicly funded botanical gardens in different biogeographic regions of India. Majority of the botanical gardens are used for recreational purposes, but only a few have focused for biodiversity conservation of especially rare, endangered, threatened, and endemic flora (Annon 2007). Arboretum generally refers to a place established for conservation of tree species. The Regional Plant Resource Centre, Bhubaneswar, India, was established as an arboretum for 1430 species of trees, 29 species of endemic/rare/threatened/endangered plants to Eastern Ghat region, a palmetum (38 species), a bombasatum (34 species), orchidarium (220 species), and cactus house (1050 species).
6. Clonal repositories: The economically potential crops, which are perennial and commercially propagated through vegetative methods, are conserved in clonal repository. Clones should be preserved by keeping living plants in orchards and field collections, in greenhouses, and in screen houses or in in vitro as tissue cultured plants and as meristems or other plant parts in cryogenic preservation (Hummer and Reed 1999).

This system provides an opportunity for limited characterization, multiplication, conservation, and supply of the genetic resources.

Table 3.4 Designation of repositories for different biological resources[a]

S. no.	Name of the institution	Category of biological resources
1	Indian Agricultural Research Institute, New Delhi	Fungi/microbes
2	Institute of Microbial Technology, Chandigarh	Microorganisms
3	Wildlife Institute of India, Dehradun	Fauna
4	National Bureau of Fish Genetic Resources, Lucknow, Uttar Pradesh	Fish
5	Zoological Survey of India, Kolkata	Fauna
6	Forest Research Institute, Dehradun	Flora
7	National Bureau of Plant Genetic Resources, New Delhi	Plant
8	National Institute of Virology, Pune	Viruses
9	National Bureau of Agriculturally Important Microbes, Mau Nath Bhanjan	Beneficial microorganisms
10	National Institute of Oceanography, Goa	Marine flora and fauna
11	National Bureau of Animal Genetic Resources, Karnal	Domestic animals
12	Tropical Forest Research Institute, Jabalpur	Moth, termites, and butterflies
13	National Botanical Research Institute, Lucknow	Flora
14	Botanical Survey of India, Kolkata	Flora
15	Institute of Forest Genetics and Tree Breeding, Coimbatore	Flora

[a]Anonymous (2007)

Needless to prove that biodiversity is the backbone of sustainable agriculture and food security and an important factor for the conservation of the environment as a whole. However, the loss of biodiversity from the agroecosystem is mainly due to the destruction of habitat, reduction of genetic diversity, introduction of alien species, decline of forest area, change of climate, and desertification and unscientific harvest of resources. Particularly in India, agro-biodiversity needs an urgent intervention to conserve of the valuable genetic resources for perpetuity, sustainable use, and development which will ensure better livelihood and will reduce the potential impact of climate change.

In India, conservation efforts were initiated mainly by various public organizations, and around 13 institutions are acting as repositories for different biological resources under the aegis of Biological Diversity Act, 2002 (Table 3.4).

3.3 High-Throughput Molecular Tools for Management of Plant Genetic Resources (PGRs)

PGRs represent agro-biodiversity and are considered to be the backbone of plant breeding. Therefore, a sustainable utilization of PGR is prerequisite for the development of different varieties which can be achieved by preserving the existing genetic

diversity of the crop species and their application in genomics studies. However, the major bottleneck is the redundancy that remains in germplasm collections which always create problem in plant improvement program. Traditionally, this was checked by using various DNA-based markers, though, recently, it is dealt with high-throughput sequencing technology without any shortcomings.

Conventionally, large numbers of genotypes are planted under different agroclimatic zones for their performance for yield or biotic or abiotic stress tolerance or specific agroclimatic zone to evaluate the performance for a specific trait. Once proven suitable across different generations in the case of cereal or several years for perennial crops, that particular genotype is considered for future breeding material for developing the cultivars. Further to assist the conventional breeding, various physiological tools and molecular markers are used. This not only reduces time but also enhances the accuracy. Several cultivars of various crops have been developed using this approach and contributed significantly in Indian agriculture including green revolution. However, with the advancement of science, aspects of PGR management have gained momentum in terms of handling large number of PGRs at a time to detect the variation with high accuracy.

Sequencing of a large number of germplasm collections for eliminating the duplicated sample is practically not feasible. Hence, the alternative is to select small representative of the entire germplasm with maximum diversity which is known as "core germplasm." With high-throughput sequencing, it is now feasible to resequence hundreds of individual plants at whole genome level. Thus, genomic data along with taxonomic, phenotypic, and ecological data will render better management of gene bank for the valorization of PGR. Hence, application of NGS will have great impacts not only on PGR conservation and management but also on their utilization in genomics-based application in breeding.

Recently developed high-throughput technologies of PGR management are the following.

3.3.1 Sequence-Driven Conservation

Next-generation sequencing (NGS) is an efficient tool to characterize PGRs. Occurrence of redundancy among the *ex situ* germplasm collections is an unavoidable situation. Identifications of duplicated samples by DNA markers suffer due to the lack of commonality of panel of markers, mimicking the exact PCR reaction in terms of use of same brand of chemicals or instruments across the different laboratories. On the other hand, NGS data does overcome such kind of shortfalls and, therefore, are considered to be an ideal tool to identify redundancy.

However being a costly affair, sequencing the large number of genotypes to identify redundancy is not a pragmatic approach as technically it would be impossible to sequence each and every individual in a large collection of crops. Thus, developing the true representatives of the genetic diversity of the entire germplasm which is known as "core collection" is the best alternative (Glaszmann et al. 2010).

Additionally, "core collection" being lesser in number will be suitable to handle for public, standardized, and well-characterized resource for the scientific community. However, once developed, such well-characterized, multiplied, isolated core reference set (CRS) will be valuable PGRs, as it can be served as reference for comparative studies, future reanalysis, and integrative genomic analysis (Hawkins et al. 2010). High-throughput sequencing is most suitable for sequencing the core collection sets at much lower cost with low coverage that identifies the rejection of duplication and reveals the genetic diversity (Bansal et al. 2010; Davey et al. 2011). There are many high-throughput sequencing-based methods developed recently. They are low-coverage sequencing for genotyping (Andolfatto et al. 2011; Elshire et al. 2011; Huang et al. 2009), restriction site-associated DNA sequencing (Baxter et al. 2011), reduced-representation libraries (You et al. 2011), and complexity reduction of polymorphic sequences (Van orsouw et al. 2007). These all methods are suitable for the characterization of PGRs, particularly for non-model species or for potential donor plants for breeding with lower genetic polymorphism. In these cases, one does not need the prior sequence information which is why they are gaining popularity. Even these methods can be applied to identify SNP-based haplotype diversity within and between closely related plant species or within wild natural populations (Ossowski et al. 2010; Pool et al. 2010).

3.3.2 Complexity Reduction of Polymorphic Sequences (CRoPS)

In CRoPS technology, tagged complexity-reduced libraries are prepared by amplified fragment length polymorphism (AFLP) by a methylation-sensitive restriction enzyme (Davey et al. 2011). The AFLP fragment libraries are then sequenced at five- to tenfold average redundancy in micro-fabricated, high-density picoliter reactions using Genome Sequencer (GS). The generated sequences are clustered and aligned, which are then mined for SNPs using in silico analysis. The advantage of this technique is that irrespective of genome complexity and size, it can be applied to diverse organisms, particularly suitable for the organism with high level of repetitive DNA in the genome and/or lower degree of polymorphism in the breeding line without any prior sequence information. For example, using CRoPS, in maize, more than 75 % discovered SNPs were successfully validated (Van Orsouw et al. 2007).

3.3.3 Restriction Site-Associated DNA Sequencing (RAD-seq)

RAD-seq is another recently developed powerful tool for targeted sequencing particularly suitable for non-model species (Kakioka et al. 2013). RAD-seq identifies SNPs adjacent to the sites of restriction endonuclease. In this method, DNAs are

first digested with restriction enzymes, ligated with an adapter containing a molecular identifying sequence (MID) unique to each sample, and then the ligated DNAs associated with each restriction site were sequenced using the massively parallel sequencing technology (Stapley et al. 2012). Several RAD-tag markers are developed which are used for genotyping simultaneously for multiple samples. It is used for several purposes of genetic analysis particularly for orphan crops including association mapping in crop plants (Ganal et al. 2009), QTL identification in barley (Chutimanitsakun et al. 2011), analysis of linkage disequilibrium in rye (Li et al. 2011), and comparative genomics in several plant species (Ashley et al. 2012).

3.3.4 Reduced Representation Libraries (RRLs)

Its high speed and reducing cost of sequencing make RRLs a better alternative for genome-wide SNP genotyping. In this method, sequencing is done in an enriched subset of a genome by excluding a proportion of its repetitive fractions. This was first used in humans to efficiently find SNPs using Sanger sequencing (You et al. 2011). The reduction in genome complexity is done by the construction of an RRL in two main steps: restriction digestion and size selection. Fragments selected from a size-selected digestion permit a similar subset of fragments to be obtained from different genotypes that can be sequenced at higher depth for accurate SNP discovery. The advantages of this technique are that the reduction of genome complexity can be optimized by selecting different combinations of restriction enzymes or size ranges, facilitates re-sampling, and generates sufficient depth for accurate identification of SNP. In soybean (Hyten et al. 2010) and sorghum (Nelson et al. 2011), resequencing of RRLs has been performed for the identification of SNPs.

3.3.5 Genotyping by Sequencing (GBS)

GBS is a recently developed cost-effective method for simultaneous discovery of SNP and genotyping. It may be used for the discovery and identification of SNPs or as a screen for panels of thousands of known markers (Davey et al. 2011). This technique becomes important for association studies and genomics-assisted breeding for various crops including those complex genomes without a reference sequence. Although GBS is simple to perform for small-size genomes, target enrichment or reduction of genome complexity must be employed for species with large genomes to ensure sufficient overlap in sequence coverage (Elshire et al. 2011; Stapley et al. 2012). Reducing the complexity of the genome by the restriction enzymes is easy, quick, extremely specific, and highly reproducible compared to the contemporary sequence capture approaches, such as different DNA hybridization or

sequence capture methods. In this approach, repetitive regions can be ignored, and instead, lower copy regions can be targeted by appropriate restriction enzymes with much higher efficiency. It has advantages of simplifying the computationally challenging alignment problems in species with high levels of genetic diversity.

The GBS procedure was successfully used for maize and barley (Oregon Wolfe Barley, where roughly 200,000 and 25,000 sequence tags were mapped, respectively (Elshire et al. 2011)). A larger-scale GBS has also been used for soybean (Sonah et al. 2013). Around 10,120 SNPs were identified; interestingly, the distribution of these SNPs was matched closely with the distribution of gene-enriched genome region of soybean. It has been found that while 39.5 % of the SNPs were present in genic regions, 52.5 % of these were located in the coding sequence. They also demonstrated the use of selective primers to reduce greater complexity during GBS library preparation. This way a number of SNP could be increased by around 40 % with doubling their depth of coverage. This paves the way to increase in the throughput while significant decrease in the per sample cost.

3.4 Molecular Tools for Generation of Plant Genomic Resources

3.4.1 De Novo Sequencing or Whole-Genome Sequencing

In many instances, agronomically important traits are found in wild species or otherwise less important genotypes in a particular species. Perhaps the best example is "mangrove," a halophyte that grows in high-saline water (Mondal and Sutoh 2013). Thus, there is a need to sequence the genomes of such non-model plant which may not be directly related with cultivation. Till recently, the sequencing of entire genomes was tough due to high cost, low sequencing speed, time-consuming, laborious, and involvement of multi-institutional collaborations. This perhaps restricted the genome sequence projects to few model plants only. Due to the development of high-throughput sequencing technology with lower cost, time of sequencing has been reduced by several orders of magnitude, which is why there is an increasing tendency to sequence de novo wild, and underutilized species recently. In India several such crops such as neem (Krishnan et al. 2011), pigeon pea (Singh et al. 2012), and an indigenous medicinal plant, *Picrorhiza kurroa* (Gahlan et al. 2012), have recently been sequenced de novo for the first time. Genome sequence of orphan plants with the absence of a reference genome is the landmark as these genomic resources are prerequisite to harness the benefit of diversity that are found with PGRs. Considering together genome sequence of a particular plant species opens up a new area for the better utilization of PGRs of that particular species or related genera.

3.4.2 Resequencing of Whole Genome

Once the genome of a genotype is sequenced, it is used as "reference genome" for the alignment of the sequences of the other genotypes of the same or different related species, which facilitate the detection of genetic variations at the level of DNA for a large number of samples within a short period of time. Thus, introduction of high-throughput sequencing made it possible to resequence the whole genome of several individuals or "core germplasm" collections rapidly.

Additionally, this has also overcome the difficulty of ascertainment bias obtained by biparental mapping populations for the estimation of linkage disequilibrium and genetic relationships among the accessions (Cosart et al. 2011; Mondal and Sutoh, 2013; Moragues et al. 2010). The largest resequencing project of plant is "3000 rice resequencing project" that was completed in 2014 (Anno 2014). Before that, "The 1001 Genomes Project" was launched at the beginning of 2008 for discovering the variations of 1001 accessions of the model plant *Arabidopsis thaliana* at DNA level. Several *Arabidopsis* genotypes have been sequenced since then (Cao et al. 2011; Lister and Ecker 2009), indicating the majority of common small-scale polymorphisms as well as many larger In-Del markers in *A. thaliana* pan-genome. Variations are identified by comparing the mutations of *A. thaliana* that has diverged from its closest relative 10 million years ago. Subsequently, similar type of whole-genome resequencing in several crop species such as in maize (Lai et al. 2010; Xu et al. 2011a, b), rice (Xu et al. 2011a, b), *Medicago* (Branca et al. 2011), and soybean (Lam et al. 2010) has been done primarily to detect the single-nucleotide polymorphisms (SNPs).

3.4.3 Transcriptomics: Coding Genomic Resources

PGRs are the vast source of biological diversity. Therefore, to develop the plant genotypes befitting the needs of ever-changing climate and region-specific needs of agriculture, biologist put effect to identify the suitable genomic resources mainly the genes. Cloning of individual genes was tedious, has low throughput, and was expensive mainly due to technological limitations. However with the advancement of various techniques of molecular biology, it becomes easy to detect the trait-specific genes with high throughput. In many instances, a trait is governed by several genes, and therefore it is necessary to identify the entire set of genes that are responsible for that trait. Although differentially expressed genes can be detected by several ways, such as massive parallel sequence signatures, serial analysis of gene expression, differential display of reverse transcriptase, selective subtractive hybridization, microarrays, and cDNA-amplified fragment length polymorphisms (Nobuta et al. 2007), all of them, however, present merits and demerits. Nevertheless, newer techniques always have additional advantages than the older techniques. Therefore, due to the larger sensitivity of RNAseq, it is widely used for identification of differentially expressed genes.

For example, microarrays are available for model organisms only (e.g., Affymetrix offers microarrays for approximately 30 organisms only). Additionally, sequencing revealed more details about transcriptional features than arrays. Identification of novel transcripts, allele-specific expression, and identification of alternative splicing are some of the examples which array cannot do. Therefore, RNAseq has become the popular choice to identify differentially expressed genes for several traits. For example, prolonged exposure to 6–12 °C as vernalization induces reproductive growth, resulting to bolting and flowering in the sugar beet. Therefore, to find the differentially expressed transcripts during vernalization in sugar beet, RNAseq analysis was done, which leads to the conclusion that RAV1-like AP2/B3 domain-containing genes are important for vernalization (Mutasa-Göttgens et al. 2012). Genes responsible for cold acclimatization in blueberry have been identified by RNAseq which were further validated through Q-PCR analysis. Furthermore, they also developed genic SSR markers to map some candidate genes (Rowland et al. 2012). The combination of whole-genome sequencing along with SuperSAGE (a high-throughput transcriptome profiling technology) discovered the deep molecular understanding of salinity tolerance in chickpea (Molina et al. 2011). Around 9000 chickpea-expressed sequence tags along with alternative transcript isoforms were found to be involved in salt stress dynamics. Similarly, drought-responsive genes were identified using RNAseq technique in cotton (Ranjan et al. 2012), which are either not expressed or downregulated in susceptible cultivars of cotton indicating that those play a direct role in tolerant cultivars. Based on the RNAseq data, several genes and related pathways were also identified that are regulated by osmotic stress in sorghum (Dugas et al. 2011).

3.4.4 Small RNA: Noncoding Genomic Resources

Small RNAs are noncoding regulatory molecules that are involved in gene expression in every domain of life. Although different types of small RNAs have been discovered in various plants by traditional cloning, their number has increased significantly after the use of NGS due to the unique chemistry of sequencing. miRNAs undergo various steps to process mature miRNA (~22 bp long). miRNAs are also produced from the intronic regions of some genes in *A. thaliana* and rice (Meng and Shao 2012). For understanding the biological roles of miRNAs, most of the miRNA targets were predicted using bioinformatics tools based on either the perfect or nearly perfect sequence complimentarily between the miRNA and target genes or sequence conservation among different species in higher plants; however, due to the mismatches in miRNA-target pairing, it remains as a challenge. Out of the predicted targets, so far only a few targets have been validated experimentally. However, a new high-throughput technique called degradome sequencing has successfully been applied for screening miRNA targets. Many novel miRNAs with low abundance and their targets have been identified and validated using NGS in conjunction with this

technique (Addo-Quaye et al. 2008). Due to the advancement of sequencing technology and bioinformatics, the last decade has witnessed remarkable progress in our knowledge of the biogenesis and activity of diverse classes of small RNAs. For example, Moxon et al. (2008) identified several miRNAs and other small RNAs in tomato fruit and leaf using high-throughput pyrosequencing, such as SlymiR 156/157 and SlymiR160. Expression of 25 miRNA families, such as miR156, miR390, and miR529, in maize was verified by deep sequencing of small RNA libraries (Zhang et al. 2009). Conserved nature of miR444 in rice, maize, sorghum, and sugarcane was also deciphered using NGS technology. In *Medicago truncatula*, 238 potential candidate miRNAs were identified. Among them, 14 new miRNAs and 15 new members of known miRNA families, whose complementary miRNAs, were also detected (Wang et al. 2011). Combing small RNA sequencing along with degradome sequencing, a total of seven miRNAs and eight *trans*-acting small interfering RNAs in wheat have also been discovered (Tang et al. 2012). Many stress-associated miRNAs have also been discovered and validated through real-time PCR in several crop plant species. These include soybean (Kulcheski et al. 2011), rice (Cao et al. 2011), and cowpea (Blanca et al. 2011).

3.4.5 Genome-Wide SNP, SSR, or Haplotype Discovery

Allelic variations can be detected by molecular markers such as SSR, SNPs, or haplotypes (i.e., groups of SNPs that are linked to a particular trait) which is the main objective of studying genomics in PGR. SNPs are presently the marker of choice due to their abundance and stability compared to the other rapid markers, such as simple sequence repeats (SSR) (Ashley et al. 2012). SNPs have been applied in areas including marker-assisted breeding, crop improvement, conservation, construction of genetic maps, detection of genetic diversity, identification of genotype/phenotype associations, etc. In functional genomics studies, SNP analysis is mainly focused on those SNPs located within regulatory genes and transcripts. These studies have largely been improved by the automation of genome-wide SNP genotyping. Until recently, large-scale SNP discovery and analysis in plants were mostly limited to maize, *Arabidopsis*, and rice. However, due to dramatic improvement of NGS technology, large-scale SNPs have been discovered in several crops within few years. Genome-wide SNPs have been discovered in various model and crop plants, such as wheat (Trebbi et al. 2011), rice (McNally et al. 2009, Yamamoto et al. 2010), *Arabidopsis* (Jander et al. 2002; Zhang and Borevitz 2009), barley (Close et al. 2009), sorghum (Nelson et al. 2011), soybean (Hyten et al. 2010), potato (Hamilton et al. 2011), maize (Jones et al. 2009), and so on, for various applications such as genetic diversity analyses, construction of linkage map, marker-assisted selection, and association mapping.

3.5 Molecular Tools for Value Addition to PGRs

The eliteness of PGRs can only be useful with proper identification of the genotypes. However, methods of identification of elite genotypes vary from crop to crop. For example, while biparental mapping population is generated for short duration crops, natural vegetative selection, pseudo-test cross, etc., are preferred for perennial crops. Recently, association mapping becomes a favored choice as an alternative technique to the linkage mapping, as it can overcome the shortfalls of biparental-based linkage mapping by using recombination events from many lineages (Abdurakhmonov and Abdukarimov 2008). Association mapping involves unstructured populations that have undergone many recombination events (Zhao et al. 2011). Therefore, by overcoming the shortfalls of traditional biparental-based linkage mapping, association genetics are widely used to identify trait-specific QTLs/genes in many crops (Hall 2010). Furthermore, an improved version known as "nested association mapping," which has the advantages of both linkage analysis and association mapping in a single mapping population, is also being used for the genome-wide dissection of complex traits (Yu et al. 2008). Following this technique, several QTLs have been mapped, cloned, and introgressed to elite lines. Alternatively, genome-wide association mapping in a wide range of diverse genotypes offers new avenue toward the discovery of alleles with agricultural importance and also for dissecting the genetic basis of complex quantitative traits in plants (Hall 2010; Mackay et al. 2009).

Genome-wide association studies, however, demand a genome-wide analysis of genetic diversity by comparing reference genome sequence with resequenced counterparts, patterns of population structure, and the decay of linkage disequilibrium. For this, suitable genotyping techniques, phenotyping resources, saturated mapped markers, and, preferably, a well-annotated reference genome sequence are prerequisite (Rafalski 2010). For example, in maize, water stress at the flowering stage causes loss of kernel set, which leads to low yield. Therefore, an association mapping-based approach was employed to identify loci that were involved in the accumulation of carbohydrates as well as abscisic acid (ABA) metabolites during stress. Further a panel of SNPs in major genes from these metabolic pathways was used to genotype 350 tropical and subtropical inbred maize lines that were well watered or water stressed at the flowering stage.

3.6 Molecular Tools for Widening the Diversity

Mutants are the most important genetic resources that play an important role in breeding as well as in genomics studies (Henikoff et al. 2004; Till et al. 2003). Although natural variations are usually used to select the parent for developing mapping populations, however, sometimes natural variants are not available for a particular trait. Additionally, development of superior plant type is an ongoing

effort. Besides, it is easy to identify the novel variants among the controlled or structured population rather than identifying the natural variants among the vast genetic resources. Therefore, developing the mutants in plant breeding remains a very popular program from the beginning. Although physical or chemical mutagenesis contributed enormously for decades to induce mutation, several recent techniques consisting of chemical mutagenesis coupled with molecular breeding have also been developed. For example, the targeting induced local lesions in genomes (TILLING) has been developed to detect polymorphisms mainly point mutations, from an induced mutant population in a target gene by heteroduplex analysis (Till et al. 2003). It is used to find out the allelic variations before commencing with the more costly and labor-intensive phenotyping (Henikoff et al. 2004). Thus, TILLING is gaining popularity for mutation characterization (Comai and Henikoff 2006) and for analyzing SNPs (Cordeiro et al. 2006). Further, a very sensitive high-throughput screening method based on capillary electrophoresis has been developed (Cross et al. 2007). It uses endonucleolytic mutation by internal labeling which greatly improves the efficiency of this new reverse genetics approach to crop improvement. Further, a variant of this technique, EcoTILLING, is used for detecting haplotype diversity which reduces the number of genotypes for sequencing and hence becomes cost-effective and timesaving. Identification of salt tolerance-associated SNPs as well as drought tolerance-associated SNPs in rice (Naredo et al. 2009) and drought-associated SNP haplotype in barley (Cseri et al. 2011) is one of the examples that have used EcoTILLING approach, yet a large number of mutant screenings by NGS have not yet been done for identifying any kind of abiotic stress-related mutations. TILLING has shortfalls too due to the poor cleavage efficiency and 5′–3′ exonuclease activity of CEL I enzyme, which is widely used in TILLING (Till et al. 2006). Moreover, being a prescreening method, it is not possible to know the information about the nature of changes at sequence level and their possible effect on phenotype. To overcome these shortfalls, there is a need to develop a high-throughput and low-cost amplicon-based sequencing method. "Key Point technology," a high-throughput mutation or polymorphism discovery technique, is such an example which is based on 454 sequencing chemistry for amplifying the target genes from mutant or natural populations (Rigola et al. 2009). Using this technique, after screening more than 3000 M_2 families in a single sequencing run, two mutants in the tomato of *eIF4E* gene were identified. Thus, NGS is potential for the quick discovery of abiotic stress-tolerance genes.

3.7 Summary and Conclusion

India is a vast reservoir of biodiversity including agro-diversity. There are several steps taken by conservation of Indian biodiversity through governmental agencies. Plant genetic resources along with its diversity provide the platform for varietal development of crops befitting the changing climate. Although conventional

approaches are well established and contributed significantly to develop various plants, yet with the advancement of recent sequence-based technologies, various high-throughput techniques have been developed in recent past which will enhance the utilization and value addition of plant genetic resources (PGRs), along with their proper gene bank management.

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Chapter 4
Methods in Transgenic Technology

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Abstract Genetic engineering had turned into a basic instrument reproducing of yields. In the course of the most recent decade, a significant advancement has been made in new and efficient transformation strategies in plants. In spite of an assortment of accessible DNA delivery techniques, *Agrobacterium*-mediated transformation remains a prevalently utilized methodology. Specifically, advance in *Agrobacterium*-mediated transformation of grains and other recalcitrant dicot species has been very noteworthy. Meanwhile, other transgenic-empowering advancements have developed, including gateway-based method, generation of marker-free transgenics, gene targeting, and chromosomal engineering. Despite the fact that alteration of some plant species or elite germplasm remains a challenge, further headway in transformation innovation is expected on the grounds that the components of overseeing the recovery and transformation procedures are currently better comprehended and are as a rule innovatively connected to outlining enhanced transformation techniques or to growing new empowering advancements.

4.1 Biological Methods of Transformation

Attributable to the need of safe harvests for sustaining the regularly developing populace of the world, interest for enhancing the plant transformation techniques is likewise expanding at the same time. Preparatory endeavors for altering the plants were begun amid 1960s on maize (Coe and Sarkar 1966), which have opened better

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approaches to modify crops for enhanced production and improved imperviousness to the ecological burdens (Estruch et al. 1997). Aside from crop improvement, the capacity to genetically design plants is likewise a capable and educational means for considering the gene function and the regulation of physiological and developmental processes. Transgenic plants are being utilized as an assay system for the adjustment of endogenous metabolism system or gene inactivation (Hansen and Wright 1999). Research in plant transformation is focusing now, less on the presentation of DNA into plant cells, but instead additional on the issues connected with integration and reliable articulation of the DNA, once it has been immobilized into the plant genome (Newell 2000). The point of this chapter is to give a review of different strategies that are generally being utilized as a part of plant transformation.

4.1.1 Agrobacterium-*Mediated Method*

Agrobacterium-mediated genetic transformation is the most broadly utilized innovation for the production of genetically altered transgenic plants. With its expansive host on the go, this strategy is the most supported means for change of a few agronomically and horticulturally important crops (Gelvin 2003). Recombinant *Agrobacterium* strains, where the native T-DNA has been supplanted with the gene(s) of importance are most effective means for bringing outside genes into plants and subsequently adjusting them into transgenic plant species. Therefore, *Agrobacterium* science and its biotechnology has been the subject of exploration in recent decades by plant architects to develop new *Agrobacterium* strains, plasmids and conventions that are exceptionally adjusted for the genetic transformation of different plant species.

With of late advancements, the host scope of *Agrobacterium* has been further broadened from one single plant to the different non-plant species (Lacroix et al. 2005a, b) starting from the level of prokaryotes and going up to human cells, in some cases (Kunik et al. 2001). *A. tumefaciens* or *A. rhizogenes* are for the most part used to bring the foreign genes into plant cells. *A. tumefaciens* is a soilborne Gram-negative bacterium that causes crown gall, a plant tumor. The event of an extensive Ti (tumor-inducing) plasmid in virulent strains of *A. tumefaciens* empowers them to induce tumor, though the Ri (root-inducing) megaplasmids, present in the virulent strains of *A. rhizogenes*, cause "hairy-root" infection. Both plasmids contain "T-DNA" (transferred DNA), which at last contains two sorts of genes: oncogenic genes that encode proteins required in the combination of auxins and cytokinins (bringing on tumor arrangement) and the genes required in opine production. At the closures of T-DNA, two 25-bp direct repeats of left outskirt (LB) and right fringe (RB), separately, are available. They act as *cis* component that signals for the T-DNA exchange (Zupan et al. 2000). Both tumorigenic and opine catabolism genes are found inside the T-DNA of the Ti plasmid, though the virulence (*vir*) genes are situated outside the T-DNA on Ti plasmid and the bacterial chromosome. These *vir* genes are available onto a few operons (*virA*, *virB*, *virC*, *virD*, *virE*, *virF*, *virG*, and *virH*) on the Ti plasmid and different operons (*chvA*, *chvB*, and *chvF*) that are chro-

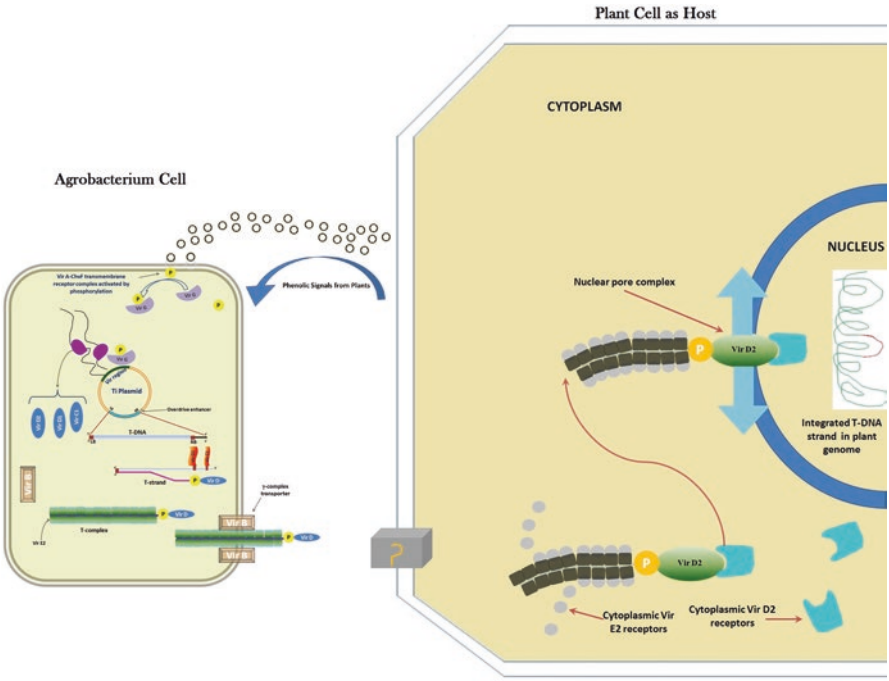

Fig. 4.1 Schematic representation of *Agrobacterium*-mediated genetic transformation

mosomal and are fundamental for the T-DNA transfer. The instrument of gene transfer from *A. tumefaciens* to the host plant cell includes various strides. These are comprised of colonization by bacteria, start of bacterial virulence framework, arrangement of T-DNA transfer complex, and T-DNA transfer took after by coordination into the plant genome (Fig. 4.1).

The bacterium receives existing cell forms (e.g., DNA and protein transport, focused on proteolysis, and DNA repair) to change its host (Tzfira and Citovsky 2002; Gelvin 2003). Also, an assortment of host proteins has been accounted for to join in, for the most part in the later stages (i.e., T-DNA intracellular transport, nuclear import, and combination) of the *Agrobacterium*-intervened genetic transformation process. The transformation procedure begins with the connection of bacterium with plant cell took after by stimulation of the expression of VirA/VirG, two-part signal-transduction system after sensing specific host signals (e.g., phenolic compounds) obtained from the host cells. Prior reports propose that injuring and/or energetic cell division also promote T-DNA transfer, most presumably because of provocation by phenolic mixes produced amid the cell repair or in the development of new cells. Taking after the initiation of vir gene region, a flexible copy of single-stranded T-DNA particle (T-strand) is then created by scratching both left and right borders on the base strand of the T-DNA with the joined activity of bacterial VirD1/D2 protein complex (Filichkin and Gelvin 1993). The T-DNA exists as a ssDNA–VirD2 protein (immature T-complex) molecule covalently appended to the 5′ end of

the T-DNA strand (Ward and Barnes 1988). This complex, together with a few other Vir proteins (Vergunst et al. 2000), is traded into the host-cell cytoplasm through the channel framed by the VirB/D4 type IV secretion system (Christie 2004, 1997), which requires a correspondence of bacterial T pilus with no less than a solitary host-specific protein (Hwang and Gelvin 2004). Prior to its entrance into the host cytoplasm be that as it may, the immature T-complex is covered by VirE2 proteins, shaping a full-grown T-complex. These VirE2 proteins are the single-stranded DNA-restricting *Agrobacterium* proteins which are shipped into the host plant cell, where they shield the mature T-complex from degradation while traveling to the host-cell nucleus. Amid the last strides of the transformation procedure, the *Agrobacterium* uses different cell systems to perform the hereditary change of its host.

It is likely that the T-complex is transported to the core of the cell with the assistance of host intracellular transport apparatus, for example, a cellular-motor-assisted mechanism. In an as of late proposed system, a dynein-like *Arabidopsis* protein (DLC3) combined with another protein, VIP1, is required for the coordinated development in the intracellular transport of the *Agrobacterium* T-complex (Tzfira et al. 2002; Salman et al. 2005) toward the nucleus. As of late, an extra *Arabidopsis* protein, VIP2 (VirE2 interacting protein2), has been exhibited to assume a noteworthy part in T-DNA integration into the plant genome. The cellular association of the radial microtubules in plant cells, situated with their minus-end toward the nucleus, further helps *Agrobacterium* utilize the yet unidentified dynein-like plant engine to convey the T-complex into the nuclear pore. The extensive size of the full-grown T-complex proposes a dynamic system for its nuclear import, in all likelihood by the nuclear-import machinery of the host cell (Abu-Arish et al. 2004). For sure, both of the T-complex protein segments VirD2 and VirE2 were found to interface with host proteins for their nuclear import in host cells. VirD2 associates with AtKAPα, which mediates its nuclear import (Ballas and Citovsky 1997), while VirE2 connects with the plant VirE2-collaborating protein 1 (VIP1; Tzfira et al. 2001). Both these particles go about as molecular adaptors among VirE2 and the host-cell karyopherin α, empowering VirE2 to be “piggy sponsored” into the host-cell core (Lacroix et al. 2005a, b).

Inside the nucleus of host cell, the T-complex has two objects to perform. To begin with, it needs to move toward the incorporating point, which is available at host genome, and, second, to get isolated from its accompanying proteins before its integration. The related accomplices of T-complex, for example, VIP1, CAK2M, TATA-box binding protein (TBP), and different individuals from the host machinery, propose that they may help in directing the T-complex toward the site of incorporation in the host chromatin. Despite the fact that the inclusion of CAK2M and TBP in the change procedure is still vague, the collaboration of VIP1 with H2A histone proteins supports the recognition that *Agrobacterium* utilizes the fondness of VIP1 and other transcription components for the plant chromatin to focus on the T-complex at the site of integration. Besides, natural confirmation demonstrates that *Agrobacterium* saddles the plant-targeted proteolysis machinery to isolate the T-strand from its related proteins. This focused system is because of the ability of

VirF to target VirE2 and VIP1 for degradation in yeast cells and thus, advancing the destabilization of VIP1 in host cells and by the negative impact of a proteasomal inhibitor on T-DNA expression in planta (Tzfira et al. 2004).

To accomplish the efficient *Agrobacterium*-mediated T-DNA transfer, a few components must be thought about, including the plant genotypes, sources of explants, *Agrobacterium* strains, medium salt potency and pH, span and temperature of *Agrobacterium*–explant associations (inoculation and cocultivation), and utilization of T-DNA-inducing compounds (Cheng et al. 2004; Opabode 2006). *Agrobacterium*-mediated transformation of higher plants is presently settled for dicotyledonous species. An assortment of explants can be utilized as target materials for *Agrobacterium*-intervened transformation, including embryonic cultures, immature or mature zygotic developing embryos, full-grown seed-derived calli, meristems, shoot apices, essential leaf nodes, excised leaf blades, roots, cotyledons, stem fragments, and callus suspension cultures. In recalcitrant explants, transformation competence can be improved with the assistance of phytohormone medications. The calli demonstrated higher transient GUS activity, when picloram was utilized as a part of *Typha latifolia* (Nandakumar et al. 2004). Likewise, Herath et al. (2005) reported that in *Hibiscus cannabinus* pre-culturing of explants for 2 days with benzyladenine (BA) containing medium enhanced the transient GUS expression. The incitement of cell division by phytohormones recommends that proficient *Agrobacterium* transformation may happen at a specific phase of the plant cell cycle (Chateau et al. 2000).

Wounded tissue locales promote *Agrobacterium* cells to come into close contact with the plant cells thereby enhancing the T-DNA transfer. For instance, "dip-wounding," which is pre-injuring of the explants before cocultivation with a blade submerged in *Agrobacterium* suspension, expands change recurrence as high as tenfold. At the point when "dip-wounding" along with the utilization of phenolic compounds in inoculation and cocultivation media, the fascination of *Agrobacterium* is apparently upgraded at injured destinations, which encourages expanded access of microbes to plant cells.

The transformation recurrence *in planta* is thought to be combined with cell division or dedifferentiation of the host explants. Recent studies have uncovered the transformation skill of the cells is high in S and G2 stage/M stage compared to lower in G0 and G1 stages (Arias et al. 2006; Grafi 2004). A cell cycle study recognized the RepA, HP1, E2Fa, CycD3, and CycD1 genes to be required in the pluripotency of cells in G1-S stage and further movement through S and G2 stages (De Veylder et al. 2002). Gordon-Kamm et al. (2002) had exhibited considerable change in maize transformation by overexpressing RepA gene. Plants which express more elevated amounts of histone H2A in tissues that are more susceptible to *Agrobacterium* contamination are crucial for T-DNA joining in somatic cells (Mysore et al. 2000). Co-overexpressing E2Fa together with its dimerization accomplice, DPa, brought about expanded cell multiplication in cotyledons, prompting around double the quantity of cells as wild type (Vlieghe et al. 2003).

Agrobacterium rhizogenes strains contain a T-DNA region situated on the Ri plasmid that conveys qualities required in root start, which are fundamental for

generation of hairy roots. Studies on the capacity of Ri T-DNA-encoded genes utilizing the agropine-type Ri plasmids (Slightom et al. 1986) have prompted the recognizable proof of 18 ORFs, including rolA, rolB, rolC, and rolD genes. It was apparent that these genes additionally take part in the generation of hairy roots. *A. rhizogenes*-mediated root transformation has received significantly less consideration than *A. tumefaciens* transformation. The primary explanation behind this is the trouble in recovering plants from hairy roots transformed by *A. rhizogenes*. Subsequently, this delivery framework has been transcendently used to create transgenic hairy roots for transient measures. A standout among the most developed frameworks in such sort of measure is the creation of "composite plants" (Collier et al. 2005). The essential trademark highlight of hairy roots is their capacity to grow in plant without hormone media. These developmental qualities have made hairy roots a helpful device for secondary metabolite production, use in metabolic designing, and investigations of root biology in general (Giri and Narasu 2000; Georgiev et al. 2007). It has been shown that hairy-root cultures can be adjusted for T-DNA activation labeling studies (Seki et al. 2005). Hairy roots are additionally used to genetically explore root–bacterial communications in soybean. For instance, different studies have uncovered that hairy roots got from different soybean cultivars keep up their related nematode resistance or susceptible phenotypes (Narayanan et al. 1999). As of late, hairy roots have been utilized to produce recombinant proteins (Skarjinskaia et al. 2008). This framework is additionally reasonable for high-throughput examination of root-related *trans*gene expression in *Medicago* root tissues and is presently anticipated that would be connected to root transformation for high-throughput practical investigation of certain gene expressions in other plant species (Kuster et al. 2007).

To put it plainly, *Agrobacterium*-mediated transformation has been effectively utilized as of late in transformation of both dicots and especially monocots, the last of which had for quite some time been thought to be not able to host *Agrobacterium*. These triumphs are inferable from the advancement of the superbinary vector, the utilization of cell reinforcements, and improvement of the arrangement of inoculation and cocultivation media. This pattern of accomplishment in transformation of different plant species will proceed not as a result of new thoughts and methodologies in enhancing *Agrobacterium* transformation, additionally because of the undeniable preferences of such a characteristic gene delivery system.

4.1.2 Gateway Technology

Gateway cloning innovation offers a quick and dependable high-throughput, restriction-enzyme-free cloning procedure for plasmid construction. The Gateway technology depends on the site-particular recombination response mediated by bacteriophage λ DNA fragments flanked by recombination sites (att). These sites can be moved into vectors containing compatible recombination destinations (attL attR or attB, attP) in a reaction mixture mediated by the Gateway Clonase mix (Karimi

et al. 2002). The foundation of all depicted Gateway-compatible plant transformation vectors is the plasmid pPZP200 (Hajdukiewicz et al. 1994). Two recombination responses, catalyzed by LR and BP recombinases (clonases), separately are utilized as a part of Gateway cloning. The initial step, catalyzed by LR, embeds the gene of interest into the Gateway vector at the attL and attR locales. The subsequent construct is known as the entry clone. All entry clones have attLs flanking the gene of interest. These are vital in the Gateway framework, on the grounds that attL sites are sliced to form sticky ends by the Gateway Clonase. These sticky ends match with the sticky ends of the destination vector, which contains attR restriction sites. This procedure is called LR reaction and is intervened by LR Clonase mix, which contains the recombination proteins vital for excision and incision. The item shaped in the LR reaction is known as the expression clone, which corresponds to a subclone of the beginning DNA sequence, accurately situated in a new vector backbone. The second Gateway step is the BP response, which is the converse of the LR reaction. In the BP reaction, the DNA inserts flanked by 25 bp attB sites are transferred from the expression clone into a vector given by a plasmid containing the attP locales. The last product is termed the destination clone and contains the transferred DNA sequence. Then again, these two consecutive steps can be turned around to meet the specific cloning needs. The BP reaction in this way permits quick, effective, directional PCR Cloning (Karimi et al. 2005, 2007).

With advances in Gateway technology, it is conceivable to develop numerous vectors with fused or specifically connected multiple transgenes (Chen et al. 2006). Curtis and Grossniklaus (2003) had grown new Gateway vectors by including stop codons (in all the 3 reading frames) that are available alongside the 3′ end of a Gateway cassette (Hartley et al. 2000) that can unreservedly be transferred to all vectors inside the arrangement. As of late, for RNA interference, a high-throughput cloning system has been outlined utilizing pHELLSGATE vectors. In these vectors, the recombination sites don't influence gene silencing performance rather than the routine restriction enzyme cloning vectors. In *Arabidopsis*, overexpression of vast DNA pieces was observed to be effective utilizing Gateway technology. Molecular investigations of T_0 and T_1 generations affirmed that Gateway-compatible constructs were dynamic in addition to the *att* recombination sites which did not hinder transgene action or interfere with enhancer action in vivo (Helliwell and Waterhouse 2003; Daxinger et al. 2008). However, the utilization of Gateway-RNAi transformation vectors can be a test because of the necessity for producing double-stranded RNA through inverted repeats. In this manner, as the *att* recombination sites should be duplicated, there is an expanded possibility of changing the orientation of the intron spacer whose splicing effectiveness might be antagonistically affected, bringing about diminished RNAi-mediated silencing viability. An answer for this issue is to utilize a double-intron spacer in the inverse orientation (Heilersig et al. 2006), though the viability of such a design needs to be confirmed through from more experiments on various plant species. Recently, the "pEarleyGate" vectors have been intended for *Agrobacterium*-mediated plant transformations. These vectors translationally intertwine FLAG, HA, cMyc, AcV5, or tandem affinity purification epitope labels onto target proteins, with or without an adjoining fluorescent protein

(Earley et al. 2006; Nakagawa et al. 2007). A high-throughput protocol has as of late been developed utilizing the Gateway binary vector R4pGWBs for change of *Arabidopsis thaliana*. This vector was designed for the one-step construction of chimeric genes between any promoter and cDNA (Nakagawa et al. 2008). Autofluorescent protein tags are additionally valuable because of their capacity to visualize cellular processes in vivo (Curtis and Grossniklaus 2003). For instance, the pSAT vectors are useful for both autofluorescent protein labeling and various gene transfers (Tzfira et al. 2005). In numerous plant research laboratories, Gateway binary vector innovation has empowered *in planta* expression of recombinant proteins combined to fluorescent labels (Zhong et al. 2008). Efficient expression of fluorescent protein has been accomplished in *Nicotiana benthamiana* (Martin et al. 2009). For simple control and productive cloning of DNA fragments for gene expression studies, another Gateway expression vector has been created by joining the Gateway system and a recombineering framework. The recombineering system utilizes bacteriophage-based homologous recombination as a part of which genomic DNA in a bacterial artificial chromosome (BAC) is adjusted or subcloned without restriction enzymes or DNA ligase (Copeland et al. 2001), in this manner allowing the direct cloning of gDNA pieces from BACs to plant transformation vectors. The construct is then converted into a novel Gateway expression vector that joins related 5′ and 3′ regulatory regions, utilizing recombineering, to supplant the intervening coding area with the Gateway destination cassette. Utilizing this methodology, proficient change has been accomplished in *Arabidopsis* plant (Rozwadowski et al. 2008).

4.1.3 Native Gene Transfer Method

Transformation of native genes (including regulatory components) into plants lacking a selectable marker is exceedingly alluring to overcome consumers' worries about GM crops. Truly, the advancement of transgenics with vital agronomic traits relied on upon the use of genes derived from different living beings. Over the past decade, yet, quick advances in plant molecular biology have brought about a noteworthy shift from bacteria and viruses to the plants as essential gene sources. An expansive assortment of plant genes connected with agronomically vital attributes has now been distinguished (Rommens 2004). For instance, plants having tailored acetolactate synthase (ALS) gene showed the same elevated amounts of sulfonylurea resistance as transgenic plants that expressed bacterial ALS gene (Bernasconi et al. 1995). In the same manner, the event of glyphosate resistance in a goosegrass (*Eleusine indica*) biotype has been connected with a mutated 5-enolpyruvylshikim ate-3-phosphate synthase (*EPSPS*) gene (Baerson et al. 2002). As for native gene transfer examination, the purpose of transposon labeling and guide-based cloning techniques has brought about the recognizable proof of more than 50 practically dynamic resistance (R) genes, several of which are being utilized as suitable options to foreign antimicrobial genes in crop improvement programs. A standout among

the most agronomically imperative R-genes segregated is the RB gene from *Solanum bulbocastanum*, which gives resistance to the potato late blight fungus, *Phytophthora infestans* (Song et al. 2003).

Rapid advance has additionally been made in the development of plant-based gene substitutes and recuperation of different insecticidal proteins that are included in insect resistance. Pechan et al. (2002) in his study proposed that a maize cysteine protease of 30-kDa can be utilized to improve the maize resilience to caterpillars and armyworms. Then again, silencing or overexpressing the targeted biosynthetic genes can improve a plant's capacity to produce insecticidal supplementary metabolites. In tobacco, repression of a P450 hydroxylase gene brought about a 19-fold increment in cembratrieneol levels in trichomes, drastically improving the aphid resistance (Wang et al. 2001). Around 40 different plant genes have been used to enhance resilience to abiotic stresses. In *Arabidopsis*, overexpression of C-repeat-binding transcription factor (CBF) expanded the survival resistance to freezing, dry spell, and salt (Kasuga et al. 1999).

Different strategies, for example, promoter trapping and RNA fingerprinting, have encouraged the identification of many plant promoters (e.g., ubiquitin and actin gene promoters). Most of them contain regulatory elements that support high-level gene expression in majority of the tissues of the transgenic plants. Utilization of the identical genetic materials accessible in plants benefits genetic engineering methodologies into existing plant breeding programs.

4.1.4 Chloroplast Transformation

In genetically altered plants, the gene of interest as a rule incorporates into the nucleus. However, it is additionally conceivable to transfer the gene into the plastid. The chloroplast genome is profoundly conserved among plant species and regularly comprises of double-stranded DNA of 120–220 kb, organized in monomeric circles or in linear molecules. In most higher plant species, the chloroplast genome has two comparable inverted repeats (IRA and IRB) of 20–30 kb that separates a substantial large single-copy (LSC) region and a small single-copy (SSC) region (Lilly et al. 2001). Both the microprojectile and the protoplast-mediated transformation strategies are equipped for delivering DNA to the plastids (Maliga 2004); yet, to accomplish effective transformation, chloroplast-specific vectors are required. The fundamental plastid transformation vector is involved chloroplast- specific expression cassettes and target-specific flanking sequences. Integration of the transgene into the chloroplast happens by means of homologous recombination of the flanking sequences utilized as a part of the chloroplast vectors (Verma and Daniell 2007). The first fruitful chloroplast transformation with *aada* gene, which presents spectinomycin resistance, was reported in *Chlamydomonas* (Boynton et al. 1988). In higher plants, plastid transformation has been proficient in tobacco with different foreign genes. As of late, a few crop chloroplast genomes have been successfully transformed through the process of organogenesis, and maternal inheritance has

been watched (Lee et al. 2006). In economically important crops, for example, cotton, efficient plastid transformation has been accomplished through somatic embryogenesis by bombarding embryogenic cell cultures. Several transgenes built through chloroplast transformation have given significant agronomic attributes in plants, including insect and pathogen resistance, drought and salt resistance. In soybean expressing the Cry1Ab gene, insecticidal movement against velvet bean caterpillar was presented to the transformed plants. Progression in chloroplast engineering has made it conceivable to utilize chloroplasts as bioreactors for the generation of recombinant proteins and biopharmaceuticals. Since plastid genes are maternally inherited, transgenes embedded into these plastids are not scattered by pollen. Additionally, the transformation system also incorporates the capacity to express several genes as a polycistronic unit, thus possibly wiping out the position effect and gene silencing in chloroplast genetic engineering (Kumar et al. 2004; Daniell et al. 2005).

4.2 Physical Methods of Transformation

4.2.1 Microprojectile Method or Biolistic Transformation

Microprojectile bombardment is one of the direct gene transfer methods for improvement of transgenics. This strategy was developed in 1980s to genetically engineer plants that were recalcitrant to transformation with *Agrobacterium*. Subsequently, the technique has been broadly used to deliver transgenic plants in an extensive variety of plant species (Breitler et al. 2002). The primary particle delivery method was developed by Sanford and collaborators. DuPont extensively altered Sanford device to produce the PDS-1000/He machine. The system includes coating microcarriers (gold or tungsten particles approx 0.6–1.0 mm in width) with the DNA of interest followed by accelerating them at high speeds to enter into the cell of any life form (Fig. 4.2). Quickly, the microcarriers are spread equitably on circular plastic film (macrocarrier), and after that the whole unit is set underneath the rupture disk in the principle pressure chamber of the biolistic device. An assortment of rupture disks are accessible that burst at pressures extending from 450 to 2200 psi. Beneath the macrocarrier is a stopping screen, in which a wire mesh is intended to hold the macrocarrier, while permitting the microcarriers to go through. The target tissue is set beneath the launch assembly unit. Under a fractional vacuum, the microprojectile is fired, and helium is then allowed to fill the gas-acceleration tube. The helium pressure develops behind a rupture disk, which bursts at a particular pressure, as a result discharging a shock wave of helium that forces the macrocarriers down onto the stopping screen. The microcarriers, hence, depart the circular plastic film (macrocarrier) and keep flying down the chamber to hit and enter the target tissue, therefore conveying the DNA (Fig. 4.2).

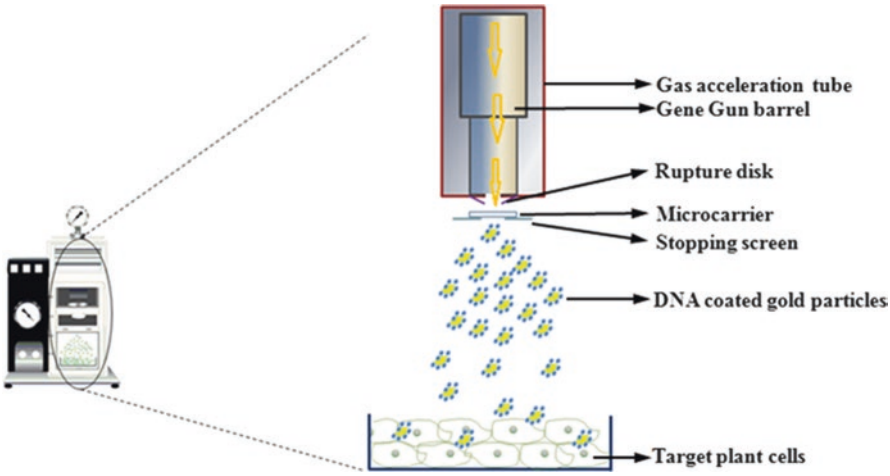

Fig. 4.2 Schematic representation of microprojectile bombardment of a plant tissue for genetic transformation

There are several variables that must be considered for an effective gene transfer using particle bombardment technology. These components consist of the configuration of a reasonable vector with a small size and high copy number and additionally the amount and nature of the DNA to be delivered. The whole procedure must be performed under strict sterile conditions to counteract the contaminations of target tissue amid consequent tissue culture. The microcarrier types and their sizes are essential decisions, since they influence the profundity of penetration of the accelerated microcarriers and also the degree of harm to the aimed cells. Gold particles ranging from 0.6 to 3.0 mm in diameter are commercially available. The level of infiltration required will rely on upon the thickness of the cell wall, the type of tissue being altered, and the profundity of the target cell layers. Variety in the helium pressures, level of vacuum created, size of particles, and the position of target tissues will direct the momentum and penetrating power at which the microprojectiles strike the tissue. All these parameters must be under the experimenter's control and should be optimized for a given target tissue (Taylor and Fauquet 2002).

Treatment of the targeted tissues before and after particle bombardment significantly affects the recurrence of recoverable transgenic cell lines and plants. An alluring component of particle bombardment is its ability to transfer alien DNA into any cell or tissue whose cell wall and plasma membrane can be penetrated. Embryogenic and meristematic tissues are the most usually utilized target tissues for the production of genetically altered plants. Particle bombardment on embryogenic tissues has been effectively exploited to generate transgenic plants in an extensive variety of agronomically essential plants, including legumes, tuber crops, starchy staples, cash crops, trees, and major cereals (Christou 1995; Campbell et al. 2000). While if the bombarding is on apical meristems, the treatment, physiological status, and age of the mother plants prior to excision of the explants must be thought

about. Utilization of an osmotic pretreatment or partial drying of the target cells preceding the particle shower is a normally utilized approach to elevate the recurrence of effective transformation (Schmidt et al. 2008).

One of the benefits of particle shower is the conceivable expression of numerous transgenes in the target tissue, which can be accomplished by the combination of genes inside the same plasmid. In recent years, different autonomous gene expression cassettes have been effectively transferred via particle bombardment in wheat, rice, and soybean (Campbell et al. 2000; Agrawal et al. 2005; Schmidt et al. 2005). The utilization of microprojectile bombardment has made simple to transfer large DNA fragments into the plant genome, despite the fact that the integrity of the DNA is of great concern. Incorporation of yeast artificial chromosomes (YACs) into the plant genome through particle bombardment method has been effective with inserts of up to 150 kb (Pawlowski and Somers 1996; Kohli et al. 1999). Even though there is a requirement of further advancement, addition of large DNA fragments guarantees to be a vital apparatus in future plant research and crop biotechnology.

Precisely, over the recent two decades, numerous published studies have used microprojectile innovation for both monocot and dicot plant species. Simultaneously, a diverse scope of agronomic traits have been transferred and bestowed to crop plants by means of particle bombardment, which remains the most encouraging procedure to genetically engineer plastids. In any case, this innovation is restricted because of a few disadvantages, for example, the incorporation of multiple copies of the required transgene, besides superfluous DNA sequences that are connected with the plasmid vector. Multicopy combinations and surplus DNA can prompt to gene silencing in transformed plant. However, this issue can be overcome by exchanging the desired coding region only with its control components into the target cells of plant genome (Lowe et al. 2009).

4.2.2 Electroporation-Mediated Transformation

Electroporation-mediated transformation requires the utilization of strong electric field pulses to cells and tissues that may bring about some kind of structural modification of the cell membrane. In vitro introduction of DNA into cells is currently the most well-known use of electroporation. The procedure was initially developed for protoplast change, yet it has been appeared to work with in place plant cells too. A voltage of 25 mV and amperage of 0.5 mA for 15 min are the frequently utilized parameters. However, variables, for example, surface concentration of DNA and resistance of cells to membrane permeation, may influence electroporation effectiveness. Utilizing the electroporation technique, fruitful changes have been achieved with the protoplasts of both monocot and dicot plants. The first report of productive transgenic rice has used electroporation of DNA into embryogenic protoplasts (Shimamoto et al. 1989). Since the protoplast-to-plant regeneration system has not been created in most plant species, subsequently, the utilization of protoplasts as explants for recovery of transformants limits the employment of

electroporation for stable changes in different plant species. The electroporation of plant cells and tissues is though fundamentally the same as in its standards to the electroporation of protoplasts. This methodology consequently empowered the recuperation of the first transgenic plants in barley (Salmenkallio-Marttila et al. 1995). In sugarcane, a gene was moved into meristem tissues through electroporation-mediated transformation. While the electroporation was projected as another option to biolistics, it is not as proficient as the last mentioned. Contrasted with biolistics, it is reasonable and basic; however, the procedure has just been fruitful in a couple plant animal categories. The thick cell wall of intact tissues corresponds to key physical hindrances to electroporation.

4.2.3 Microinjection Method

Microinjection is the direct mechanical delivery method of DNA into the cytoplasm, nucleus, chloroplast, or mitochondria by means of a glass microcapillary infusion pipette. With the help of a magnifying instrument, cells (protoplasts) are immobilized in low-melting-point agar with the holding pipette and delicate suction. DNA is then infused into these organelles (Crossway et al. 1986). The microinjection strategy requires moderately costly specialized hardware for micromanipulation of particular cells under a magnifying lens and includes exact infusion of small amounts of DNA solution. This strategy is, be that as it may, an exceptionally time-consuming system. The infused cells or clusters of cells are subsequently cultured in tissue culture systems and regenerated into new plantlets. Effective recovery of microinjected rapeseed (Jones-Villeneuve et al. 1995), tobacco, and barley (Holm et al. 2000) has been accomplished. Only restricted achievement has been attained in plant transformation because of the thick cell walls of plants and, all the more challengingly, absence of accessibility of a single-cell-to-plant regeneration system in most plant species.

4.2.4 Vacuum Infiltration

Another approach to intercede the fusion of *Agrobacterium* for plant transformation is to apply a vacuum for a specific time period. Physically, vacuum produces a negative atmospheric pressure that decreases the air spaces between the cells of the tissue permitting the penetration of bacteria into the inner cell spaces. With longer time span and the higher the negative vacuum pressure, the less will be the air space inside the cell of a plant tissue, and therefore, the more will be the infiltration of the bacteria. An expansion in the pressure permits the invasion medium, including the infective transformation vector, to move into the plant tissue. The time that a plant part or tissue is presented to vacuum is crucial as prolonged exposure causes hyperhydricity. The utilization of *Agrobacterium*-mediated transformation helped by

vacuum infiltration was initially reported in 1993 while transforming *Arabidopsis* by Bechtold et al. (1993). From that point forward, several enhancements have been made to set up powerful protocols of transformation for various plant species (Charity et al. 2002; Ikram-Ul-Haq 2004; Tague and Mantis 2006; Tjokrokusumo et al. 2000; Canche-Moo et al. 2006; de Oliveira et al. 2009; Subramanyam et al. 2011). The utility of this strategy has been as of late shown fruitful results by the production of plant inferred vaccine under the present Good Manufacture Practice (cGMP) controls for human clinical trials (Huafang and Qiang 2012).

Some advantages of vacuum penetration-facilitated transformations are the generation of many independently transformed plants from a particular plant that decreases somaclonal variation and the likelihood of high-throughput testing as the procedure is quick. The strategy is also potentially valuable for alteration in recalcitrant plants to plant tissue culture and regeneration.

4.2.5 Nanotechnological Approach

Trans-genesis has many applications in plant biology, especially in cell biology and gene function studies. Importantly, it also allows the generation of plants with improved agricultural traits and activity, significantly faster than any conventional breeding practice. The technology is based on the delivery of genes of interest from a broad range of sources into a plant genome. Demands for the simultaneous transformation of a large number of genes are increasing for functional analysis of the plant genes. Transportation of genes into plant cells is more complicated due to the plant cell wall, an extra barrier in addition to the cell membrane. The plant scientists, therefore, have been exploring efficient vectors for high transformation frequency, as they often face the problems of transferring large DNA fragments and set of genes to the plant cells for high transformation. Numerous transformation techniques have been reported in the literature, but they all have one or the other limitations. For example, *Agrobacterium*-mediated transformation is an efficient process in dicotyledonous plants (dicots), whereas it is not very efficient in monocot plants and cell types (e.g., microspores), probably due to limited integration rates and technical restriction (Frame et al. 2002). Electroporation technique needs protoplasts, which are actually plant cells without a cell wall. However, the isolation of protoplasts is a tedious process and transformed protoplasts are tough to regenerate into intact plants. Consequently, even the regeneration efficiency is very low. The particle bombardment method can be used, but it too causes injury to the cells, damages the naked plasmid DNA molecules, and can deliver the genetic material with limited molecular size in to the cell, thus reducing the transformation frequency (Russell et al. 1992). Therefore, novel genetic transfer methods need to be developed to achieve efficient, cost-effective, and nondestructive gene delivery by plant biotechnologists to plants with intact cell walls.

The development of nanobiotechnology provides novel methods and protocols for transformation of cells (Chatterjee and Sarkar 2014). It includes the fabrication

and use of different nanomaterials (NMs), including nanoparticles (NPs). Nanoparticles are classified as materials in which at least one dimensions of the material are less than 100 nm in diameter. In the year 1996, the Nobel Prize winner P. Ehrlich postulated that the nanoparticles loaded with drug can be used as "magic bullet" for smart drug delivery. Nanoparticles are also becoming an area of research interest due to their unique properties, such as increased electrical conductivity, ductility, toughness, and formability of ceramics, increasing the hardness and strength of metals and alloys, and increasing the luminescent efficiency of semiconductors, paints, sunscreens, and cosmetics. They also act as key players in environmental remediation applications (Wen-Tso Liu 2006). During the past few years, there has been extensive interest in applying nanoparticles to plants for agricultural and horticultural use (Liu et al. 2002a, b, c; Pavel et al. 1999; Cotae and Creanga 2005; Pavel and Creanga 2005).

Nanoparticle gene carriers have significant advantages compared with traditional carriers. Firstly, they are applicable to both monocotyledons and dicotyledonous plants. Secondly, this type of gene carriers can effectively overcome transgenic silencing via controlling the copies of DNA combined to nanoparticles. Thirdly, nanoparticles can be easily functionalized so as to further enhance transformation efficiency. Finally, nanoparticles-mediated multigene transformation can be achieved without involving traditional building method of complex carriers.

Numerous types of materials can be used to make such nanoparticles. These include metal oxides, ceramics and silicates, magnetic materials, liposomes, dendrimers, emulsions (for a review, see Holister et al. 2003), mesoporous silica (Torney et al. 2007), starch and poly(amidoamine) dendrimer (Pasupathy et al. 2008). For successful genetic transformation, in both mono- and dicotyledonous plants, the nanomaterials should be biocompatible, biodegradable, and less cytotoxic and genotoxic. For example, GUS gene was successfully transferred and expressed in *Brassica juncea* L. cv. *Pusa Jaikisan* by using ultra size calcium phosphate nanoparticles (20–50 nm) as nonviral carrier. The transformation efficiency was found to be about 80.7 % as compared to 54.4 % by the *Agrobacterium tumefaciens* and only 8 % using the naked DNA. These results indicate that CaP nanoparticles could be used as more efficient transforming vectors in plants as compared to the *Agrobacterium tumefaciens* (Naqvi et al. 2012). Similarly, the HMG-R gene encapsulated in calcium phosphate nanoparticles was also successfully delivered and expressed in *C. intybus* plants. These developments might revolutionize the transgenic technology used in agriculture and bring about many other applications that previously remained a far-off dream into technical realization (Fig. 4.3).

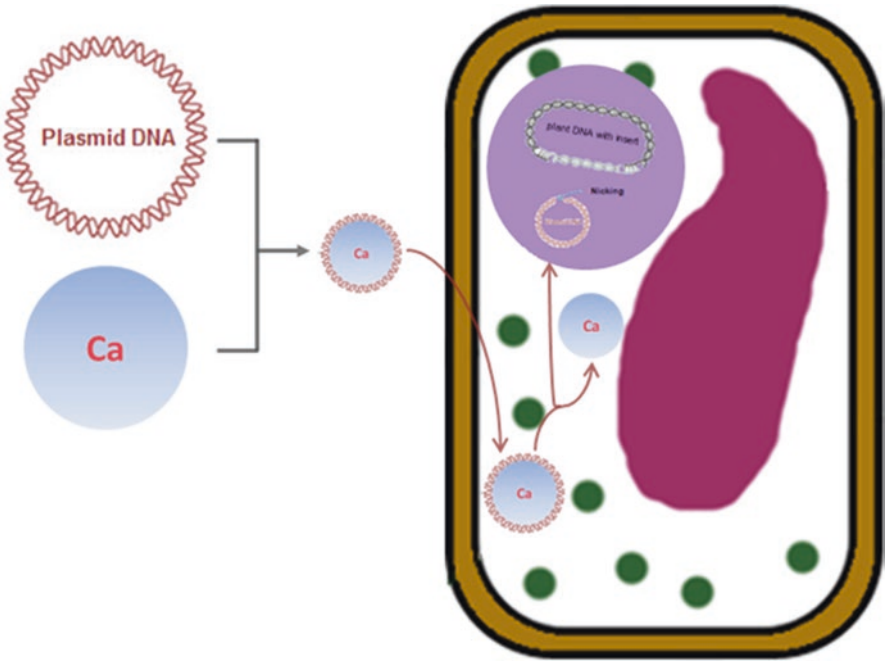

Fig. 4.3 Schematic representation of plant cell transformation employing NanoGene delivery mechanism

4.3 PEG-Mediated Transformations

Polyethylene glycol (PEG)-interceded transformation is a strategy used to deliver DNA via protoplasts as explants. This technique is much more similar to the electroporation method, where the DNA to be injected is just mixed with the protoplast and uptake of DNA is then stimulated by the addition of PEG as opposed to an electrical pulse. PEG-mediated change has several advantages, viz., it is easy to handle and no specific equipment is required, and there are few disadvantages also. The method is once in a while utilized because of the low recurrence of change and numerous species can't be recovered into the entire plants from protoplasts. Fertility of a plant can be of great concern on account of the somaclonal variation of the transgenic plants developed from the protoplast cultures. Regardless, utilizing this strategy, transgenic maize and barley have been developed (Daveya et al. 2005). In spite of fertility issues in the regenerants, protoplast transformation is possible in cereals. In cotton also transformation was accomplished utilizing consolidated polybrene-spermidine-based callus treatment (Sawahel 2001).

4.4 Liposome-Mediated Transformation

Liposome-mediated transformation strategy is closely associated with PEG transformation methods. In this strategy, DNA penetrates the protoplasts through endocytosis of liposomes. For the most part, this procedure includes three stages: attachment of liposomes to the protoplast surface, fusion of liposomes at the site of bond, and release of plasmid inside the cell. Liposomes are minuscule spherical vesicles that form when the phospholipids are hydrated. Liposomes being positively charged have a tendency to attract negatively charged DNA and cell membrane (Gad et al. 1990). In this procedure, the engulfed DNA is allowed to combine into the host genome, although there have not been many fruitful reports on the use of this procedure in plant species, as it is exceptionally laborious and the transformation frequency is very poor. In tobacco, whole YACs were altered by means of lipofection-PEG procedure (Wordragen et al. 1997).

4.5 Silicon Carbide-Mediated Transformation (SCMT)

Kaeppler et al. (1992) initially reported the utilization of silicon carbide-mediated transformation (SCMT), which is one of the minimum confounded strategies. In this strategy, little needle-type silicon carbide hairs are mixed with plant cells and the gene of interest followed by vortexing the mixture. All the while, the bristles penetrate the cells, allowing the DNA passage through the hole. The silicon carbide filaments frequently utilized have an elongated shape with a length of 10–80 mm and a diameter of 0.6 mm. They demonstrate high resistance to expandability. The technique is simple, cost-effective, and viable on different type of cells (Frame et al. 1994). The competence of SCMT relies upon fiber size, vortexing, the shape of vortexing container, and also the plant material used for transformation. The SCMT method has been utilized for transforming plants such as maize, rice, wheat, tobacco, etc. Besides, silicon carbide filaments have been found to enhance the efficiency of *Agrobacterium*-mediated transformation (Singh and Chawla 1999).

The principle shortcoming of SCMT is low transformation efficiency and cell damage, in this way influencing the regeneration capacity in a negative way. Furthermore, this technique imposes health hazards due to fiber inhalation, if not performed legitimately. More recently, two related advancements have been produced; silicon fibers and mesoporous silica nanoparticles. Silicon strands have been accounted for to expand callus transformation by 30–50 % in rice (Nagatani et al. 1997), and mesoporous silica nanoparticles have been utilized to deliver DNA and chemicals into both plant cells and leaves (Torney et al. 2007). Mesoporous silica nanoparticles are synthesized from a reaction between tetraethyl orthosilicate and a layout made of micellar bars (Nandiyanto et al. 2009).

4.6 Conclusion

The genetic modification of plants employing transgenic technology is a very important approach to regulate the production of particular metabolites, to improve adaptation to environmental stress, to improve food quality, and to increase the crop yield. Plant genetic modification requires efficient and facile gene transfer methods that will be generally applicable to as many plant species as possible. Even though the list of plant species for which transformation procedures have been established is quite extensive and encompassing over 100 different species, gene transfer into plants is by no means routine or widely available to every laboratory. With few exceptions, only a relatively small number of laboratories, mostly in the industrialized world, can perform such gene transfer experiments, particularly when one looks at the major food crops. Such difficulties reflect a multitude of complexities, some of which include biological constraints inherent in the interaction between transformation vectors and host plants, technical requirements and knowhow cost, and intellectual property. A number of different methods have been developed for the introduction of genetic materials into plants, but two techniques have dominated the field. The first is based on the ability of the soilborne bacterium, *Agrobacterium tumefaciens*, to transfer exogenous DNA into plant cells through a complex series of molecular and biochemical interactions involving signal molecules and a number of different genes residing in the bacterial cell molecular machinery. The other method relies on the physical acceleration of microscopic metal particles, viz., gold or tungsten coated with genetic material into recipient cells. These particles can be accelerated to high velocities that allow them to penetrate into target cells, where they deliver the genetic material into the nucleus, resulting in transient or stable transformation. Intact plants can subsequently be recovered from stably transformed cells. Every method has its advantages and disadvantages. However, any of these methods used by various laboratories depends largely on availability of equipments.

Even though there has been much encouraging progress with improved methods of gene containment and the real prospect of targeted transgene insertion over the past decade, a lot of work is necessary to update many of the basic technologies of transgene insertion and selection in plants, and in many cases, there is still a considerable gap between the promising lab work and its commercial development. Nevertheless, plant biotechnology remains one of the most vibrant and exciting areas of biology with immense promise to contribute to the human welfare over the coming years.

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Chapter 5
Plant Promoters: Characterization and Applications in Transgenic Technology

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Abstract Plant growth, development and adaptation are regulated by gene expression, in which promoters, the *cis*-acting elements that initiate transcription, play a pivotal role. This chapter on plant promoter characterization and applications is split into three major sections. The first section describes the structural features of plant promoters followed by their types along with examples. The understanding that promoters are a complex region of various interacting structural elements has encouraged researchers to develop synthetic promoters, and a subsection is devoted to discuss various such promoters developed. Different approaches available for promoter isolation, identification and their functional characterization are presented in the next section. This section also gives information on various mutant resources, and tools and databases available which help researchers in acquiring appropriate material for promoter isolation in model crops and carry out bioinformatic analysis of the promoter sequences/elements identified, respectively. All the related molecular techniques are also presented in brief. In transgenic development, the choice of promoter remains an important aspect which not only determines the efficiency of the transgenic crop but also might have implications in its biosafety. The third section describes various transgenic crops commercialized or in pipeline (which have completed biosafety trials) in relation to the specific promoters used in their development.

5.1 Regulatory Elements of Gene

In any multicellular organism, despite each and every cell having identical genetic material, only a small set of genes are expressed in all the cells all the time. A majority of the genes are expressed selectively in a stage-specific, development-specific and tissue-specific manner. In addition, a large number of genes are induced or repressed in response to a variety of physical or chemical stimuli. Regulation of gene expression is thus very critical for coordinated cell division, growth,

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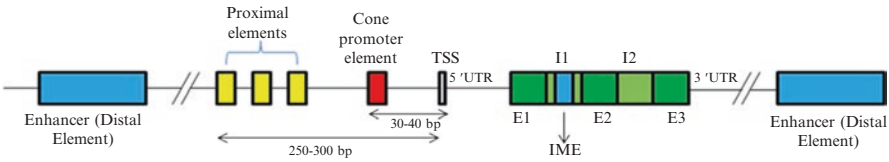

Fig. 5.1 Transcriptional regulatory elements of a typical class II eukaryotic gene; TSS *transcription start site*, UTR *untranslated region*, IME *intron-mediated enhancer*; E1, E2 and E3 are exons; I1 and I2 are introns

differentiation, reproduction and other important developmental processes. Gene regulation in eukaryotes may occur at any of the stages, namely, transcription, RNA splicing, mRNA longevity and translation, one of the most important regulatory steps being initiation of transcription of genes. One of the important parts of gene which is involved in this process is a *cis*-acting DNA element called promoter. The present chapter deals mainly with the role of promoters in transcriptional regulation in plants. Using suitable examples, the present chapter also illustrates the role and application of promoter elements in transgenics.

5.1.1 Promoter

Promoter is a *cis*-acting DNA fragment that initiates and regulates transcription of an associated gene. These DNA sequences are part of a functional gene and occur generally in the 5′ region. There are two important regions in a promoter, namely, *core* region where RNA polymerase II binds and initiates basal level transcription and *distal* region containing several *cis*-regulatory motifs for spatio-temporal control of gene expression (Rombauts et al. 2003). Core promoter is the site of action of the RNA polymerase II transcriptional machinery. Typically, the core promoter encompasses the site of transcription initiation and extends either upstream or downstream for an additional ~35 nt. Thus, in many instances, the core promoter will comprise only about 40 nt. There are several sequence motifs—which include the TATA box, initiator (Inr), TFIIB recognition element (BRE) and downstream core promoter element (DPE)—that are commonly found in core promoters (Fig. 5.1), each of them having a specific function that relates to the transcription process. It is important to note that each of these core promoter elements is found in some but not all core promoters (Butler and Kadonaga 2001).

The TATA box was the first eukaryotic core promoter motif to be identified. Though in metazoans the TATA box is typically located about 25–30 nt upstream of the transcription start site (TSS), it has a more variable position that ranges from about 40 to 100 nt upstream of the TSS in the yeast, *Saccharomyces cerevisiae*. The consensus sequence for the TATA box is TATAAA, although a wide range of sequences can function as a TATA box in vivo (Singer et al. 1990). Plant consensus

sequences for the TATA box and the region around TSSs were first explored through pioneering work by Joshi based on 79 higher plant genomic sequences from 15 different plant species (Joshi 1987). This study identified the plant consensus sequence for TATA box as TCACTATATATAG which was present in 25–39 nucleotides upstream from TSS. Initially it was believed that TATA box was present in all eukaryotic promoters, though later, with advancement in genomic studies, TATA boxes were found to be present only in a subset of core promoters. For instance, it was estimated that approximately 43 % of 205 core promoters in *Drosophila* contain a TATA box (Kutach and Kadonaga 2000). In humans, it was found that about 32 % of 1031 potential promoter regions contain a putative TATA box motif (Suzuki et al. 2001). In *Arabidopsis*, around 29 % promoters are reported to contain TATA box (~32 bp upstream of TSS) (Molina and Grotewold 2005), whereas in rice only 19 % of the promoters contain TATA box (Civan and Svec 2009). Mutations to G and C at the seventh and eighth positions in the prototype TATA box TCACTATATATAG were found to inhibit light-dependent activation of transcription, whereas substitution of T at the ninth position with G or C enhanced transcription in transgenic tobacco. In *Arabidopsis* also, the promoters containing TATAGATA, as identified by genome-wide analysis, are not tightly repressed (Ranjan et al. 2009). Conserved features in the TATA-consensus region had been identified in a dataset of highly expressed plant genes (Sawant et al. 1999). The characteristic transcription initiation site in the highly expressed genes is CAN(A/C)(A/C)(C/A)C(C/A)N_2A(C/A) with two tandem repeats of TATA elements, maximum with two point mutations. The characteristic TATA motif in the highly expressed plant genes is (T/C)(T/A)N_2TCACTATATATAG. The promoter sequence in the highly expressed genes is over-represented by C, C and G at the −3, −1 and +9 positions, respectively. Most of these features are not present in the genes ubiquitously expressed at low levels in plants.

As TATA-containing promoters are not prevalent, some other DNA elements must be responsible for coordinating transcription in a sizeable number of promoters. The initiator element (Inr), another important core promoter, encompasses the TSS and is found in both TATA-containing as well as TATA-less core promoters. The consensus for the Inr in mammalian cells is Py-Py(C)-A_{+1}-N-T/A-Py-Py (Butler and Kadonaga 2001). Though A_{+1} position is designated at the +1 start site where transcription commonly initiates, more often than not, transcription initiates either at a single site (not necessarily at the A_{+1}position) or in a cluster of multiple sites in the vicinity of the Inr. In plants, a similar pyrimidine-rich initiator motif has been defined in the promoter of the nuclear *psadb* gene encoding ferredoxin-binding subunit of photosystem I. Further computer analysis of 232 promoters revealed that majority of the photosynthetic nuclear genes lacked TATA boxes although the frequency of the TATA-less promoters in non-photosynthetic genes in plants is less than 10 % (Nakamura et al. 2002). When Inr and TATA box are present in a same promoter, they work together (Porto et al. 2013).

The BRE is located immediately upstream of some TATA boxes. The BRE consensus is G/C-G/C-G/A-C-G-C-C (where the 3′ C of the BRE is followed by the 5′ T of the TATA box), and at least a five out of seven matches with the BRE consensus

was found in 12 % of a collection of 315 TATA-containing promoters (Lagrange et al. 1998). This element has not been detected in plants either by bioinformatic or functional genomic tools. The DPE was identified as a downstream core promoter-binding site for purified *Drosophila* TFIID (Burke and Kadonaga 1996). The consensus sequence of DPE, A/G-G-A/T-C-G-T-G, is located 30 nt downstream of TATA-less TSS and has been shown to drive the expression of tungro basiliform virus promoter in transient assay of rice protoplast (He et al. 2002). TFIID binds cooperatively to the Inr and DPE motifs, as mutation of either the Inr or the DPE results in loss of TFIID binding to the core promoter (Burke and Kadonaga 1996). The DPE is found most commonly in TATA-less promoters. This motif has been well studied only in *Drosophila* but not in other eukaryotes.

A core motif with alternate purine and pyrimidine bases, CATGCATG, known as the RY repeat element, has been detected in promoters of maturing (MAT), late embryogenesis related, GA biosynthesis and seed-specific genes in both monocots and dicots. It has been shown by Reidt et al. (2000) that both the nucleotide sequence and the alternation of purine and pyrimidine nucleotides (RY character) are essential for the activity of the motif.

There is a pyrimidine-rich core element, popularly known as Y patch in plants, which is in fact more abundant than TATA box in *Arabidopsis* (Yamamoto et al. 2007). Y patch is conserved in monocots and dicots. It is exclusively present only in plants but not in animals, though its biochemical role is not yet known. TATA box and Y patch are the only direction-sensitive core promoter elements in plants. All other regulatory elements, collectively known as 'Reg' (which actually comprises of diverse *cis*-regulatory sequences), are direction insensitive (Yamamoto et al. 2007). Similarly TC motifs have been identified in rice and *Arabidopsis* 30 bp upstream to TSS. It was also observed in *Arabidopsis* that TC elements were common to genes that express only under specific condition (Bernard et al. 2010).

There are two more proximal promoter elements in the immediate vicinity of the transcription start site (roughly from − 250 to + 250 nt) which significantly affect the initiation frequency. They are CCAAT and GC boxes which are prevalent in housekeeping and TATA-less genes in animals, though no such pattern is found in plants. In *Arabidopsis*, multiple copies of genes encoding CCAAT box-binding proteins are present suggesting that they do have an important role in regulation of gene expression in plants. It is usually located close to -80 bp and can act in two directions. The GC box with the consensus sequence of GGGCGG is known to enhance the expression from four- to tenfold in mammalian genes (Nielsen et al. 1998). It often occurs in multiple copies in two orientations. Both these elements have been reported in many plant genes such as *sbeIIa* and *sbeIIb* genes encoding starch-branching enzyme (Sun et al. 1998).

In the compact region of 1000 bp upstream to the gene, various regulatory elements responding to different internal as well as external stimuli such as light, hormones, wounding, drought, heat, cold, oxidative stress and anaerobic condition with specific motifs are present (for details, refer to Komarnytsky and Borisjuk 2003 and PlantCARE database).

Among the plant promoters, there is variation in GC content, presence of transposable elements and micro- and minisatellite sequences. The GC content of the monocotyledonous promoters is higher (50.5 %) as compared to their counterparts in dicotyledons (34.1 %). Many plant promoters also harbour minisatellite and microsatellite repeat sequences which might have a role in fine-tuning gene regulation (Liu et al. 2012). For example, the most common transcription factor binding site (TFBS) motif, AGAGAGAGA, has been reported in light-responsive regulation of phototransduction in plants (Parida et al. 2009). There are also differences between consensuses of core sequence elements among plants.

5.1.2 Enhancers and Silencers

Enhancers and silencers are distal regulatory elements that dramatically enhance or repress the rate of transcription (Kolovos et al. 2012). Enhancers augment the activity of a promoter in an orientation- and position-independent manner, i.e. they do not need to be at fixed positions unlike core promoter elements and can operate in either direction, upstream or downstream to the gene (Lodish et al. 2000). The first enhancer element was described from simian virus 40 (SV40) genome (Banerji et al. 1981). Many enhancer elements have been described and experimentally demonstrated in plants such as the enhancers at −148 to −186 region of wheat high molecular weight (HMW) glutenin TSS consisting of TTGCT, a pair of GCTCC direct repeats and a sequence possessing imperfect dyad symmetry which drive endosperm-specific expression (Thomas and Flavell 1990) and AT-rich domains acting as enhancers in most, if not all, seed storage protein genes (Thomas, 1993). An enhancer (−345 to −261 region) of ST-LS1 gene in potato encoding a component of O_2-evolving complex of photosystem II was shown to induce the expression of truncated promoter even in heterologous systems (Stockhaus et al. 1989). Multiple copies of 31 bp long AT-rich sequences in the region of −444 to −177, denoted as P268 in the pea anthocyanin gene (*PetE*), had also been shown to act as strong enhancer driving expression of minimal *PetE* in tobacco roots and leaves (Sandhu et al. 1998). Pollen-specific 19 bp long enhancer elements have been shown to occur in LAT52, LAT56 and LAT59 genes in tomato involved in pollen maturation (Twell et al. 1991). Recently, the region from −1183 to −888 bp region of tapetum/microspore-specific parthenogenesis-related gene (*PR10*) from lily has been demonstrated to be highly specific to enhance expression in anther development (Hsu et al. 2014). The motif AGAAMA (where M= A or C) had been reported to occur frequently in negative regulatory sequences (silencer) in seed storage protein genes such as β-phaseolin and β-conglycinin (Thomas and Flavell, 1990). Recently, two negative regulatory regions, from −986 to −959 and from −472 to −424, harbouring TCCAAAA motif, were identified in the large subunit of ADP-glucose pyrophosphorylase isolated from watermelon and found to abolish constitutive expression but promote fruit-specific activity (Yin et al. 2009). An upstream oct-1 and oct-2 binding silencers have been identified to govern the expression of immunoglobin Igβ (Malone et al. 2000).

Introns also have been reported to harbour enhancer motifs such as those in ACT1 intron from *Arabidopsis*, AGAMOUS second introns from tobacco and UBQ10 intron from many plant species including *Arabidopsis* (Parra et al. 2011; Yang et al. 2010; Vitale et al. 2003). Enhancers are typically composed of multiple modules that work together to augment gene expression. The modules consist either of binding sites for individual transcription factors (enhancer binding proteins) or composite binding sites for different transcription factors (Grosschedl and Fiona 2001). The multiplicity and modularity of transcription binding sites in enhancers allow combinatorial control of gene expression and functional diversity. However, as compared to promoters, enhancers are imperfectly conserved showing sequence degeneracy. This makes it difficult to identify these elements through *in silico* comparative analysis. Hence to identify and verify the function and specificity of an enhancer, it needs to be identified along with its core promoter (Porto et al. 2013).

5.2 Types of Promoters

There are a total of 8301 promoter sequences available in the plant promoter database till date, rice and *Arabidopsis* comprising the bulk of these entries (PlantProm DB; http://linux1.softberry.com/berry.phtml). As predicted by the online software NSITE-PL, these entries contain 31,259 TF-binding motifs. With advances in genomics, this information would increase further. Thus it is impossible to mention/list all of them here. Hence a broad overview of different types of promoters is given below. Important promoter elements which are being actively used in transgenic development and those that have been identified after 2007 are presented in Table 5.1. Based on the nature of control of gene expression exerted by promoters, these elements can be classified as constitutive, tissue specific and temporal/stage specific or inducible. Promoters are also being designed in laboratories now for transgenic plant development which have very precise *cis* elements in repeats or in different combinations placed before the gene of interest, called as synthetic promoters. Each of these classes is briefly described below.

5.2.1 Constitutive Promoters

The constitutive type of promoters directs the expression in most or all tissues throughout the life of a plant. Their expression normally remains unaffected by endogenous factors. The constitutive promoters are usually active across species or even kingdoms. They either have *cis* elements which can interact with *trans* factors that are present in all tissues or contain multiple *cis* elements which interact with different factors in different tissues. The most widely known promoter of this type is the CaMV35S promoter. This promoter was isolated from cauliflower mosaic virus by Odell et al. (1987). It was shown to express chimeric genes constitutively in leaves, stem, root and petals of transgenic tobacco. However, its expression is often low in

Table 5.1 Promoters identified after 2007 till 2014

Promoter/cis elements	Source of identification (crop)	Tested in	Pattern of expression	References	Remarks
PvUbi1 and PvUbi2	Switch grass (*Panicum virgatum*)	Switch grass, rice and tobacco	Constitutive	Mann et al. (2011)	–
APX, PGD1 and R1G1B	Rice	Rice	Constitutive	Park et al. (2012)	–
SUI1 gene	Pineapple	*Arabidopsis*	Constitutive	Koia et al. (2013)	–
pBdEF1α and pBdUBI10	*Brachypodium distachyon*	Maize	Constitutive	Coussens et al. (2012)	–
ribosomal protein L36 gene	Pineapple	*Arabidopsis*	Constitutive	Koia et al. (2013)	–
GmERF family (four promoters)	Soybean	Lima bean and soybean	Constitutive	Hernandez-Garcia et al. (2010)	–
Gmubi family(seven promoters)	Soybean	Lima bean and soybean	Constitutive	Hernandez-Garcia et al. (2010)	–
FSgt-PFlt	Figwort mosaic virus	Tobacco, *Arabidopsis*, petunia, tomato and spinach	Constitutive and inducible by SA and ABA	Acharya et al. (2014)	Chimeric promoter
PFlt-UAS-2X	Peanut chlorotic streak caulimovirus	Tobacco, *Arabidopsis*, petunia, tomato and spinach	Constitutive and inducible by SA and ABA	Acharya et al. (2014)	Chimeric promoter
MSgt-P	Mirabilis mosaic virus	Tobacco, *Arabidopsis*, petunia, tomato and spinach	Constitutive and inducible by SA and ABA	Acharya et al. (2014)	Chimeric promoter
OsNCED3	Rice	Rice	Constitutive and stress inducible	Bang et al. (2013)	No expression in flowers and grains

(continued)

Table 5.1 (continued)

Promoter/cis elements	Source of identification (crop)	Tested in	Pattern of expression	References	Remarks
MSgt-FSgt	Mirabilis and figwort mosaic virus DNA	Tobacco	Constitutive	Kumar et al. (2011)	Recombinant
Rab21 and Wsi18,	Rice	Rice	Drought inducible in leaves and flowers	Yi et al. (2010)	–
Lea3 and Uge1	Rice	Rice	Drought inducible in leaves	Yi et al. (2010)	–
Dip1 and R1G1B	Rice	Rice	Drought inducible in all tissues	Yi et al. (2010)	–
NtHSP3A and NtHSP3B	Tobacco	Tobacco	Heat inducible	Navarre et al. (2011)	–
Vacuolar H+− pyrophosphatase (TsVP1)	*Thellungiella halophila*	Tobacco (transient assay)	Salt inducible	Sun et al. (2010)	–
AgMT3 (metallothionein 32)	Celery	*Arabidopsis*	Salt inducible	Landouar-Arsivaud et al. (2011)	–
AgMaT3 (mannitol transporter)	Celery	*Arabidopsis*	Salt inducible and root specific	Landouar-Arsivaud et al. (2011)	–
pOs03g01700 and pOs02g37190	Rice	Rice	Root specific	Li et al. (2013)	–
pMe1 (unknown function)	Cassava	Carrot	Root specific	Arango et al. (2010)	–
pDJ3S storage protein dioscorin 3)	*Dioscorea japonica* (yam)	Carrot	Root specific	Arango et al. (2010)	–
Glutamic-acid-rich protein Pt2L4	Rice	Cassava and carrot	Root specific	Beltran et al. (2010)	–
Figwort mosaic virus promoter	Figwort Mosaic Virus	Soybean	Root and root nodule specific	Govindarajulu et al. (2008)	–
AgMT2 (metallothionein 2)	Celery	*Arabidopsis*	Phloem specific	Landouar-Arsivaud et al. (2011)	–

Phloem protein 2 (CsPP2)	Citrus	Citrus	Phloem specific	Miyata et al. (2013)	–
Phloem protein 2 (AtPP2)	*Arabidopsis thaliana*	Citrus	Phloem specific	Miyata et al. (2013)	–
Sucrose transporter 2 (AtSUC2)	*Arabidopsis thaliana*	Citrus	Phloem specific	Miyata et al. (2013)	–
RTBV promoter	Rice tungro bacilliform virus	Citrus	Phloem specific	Dutt et al. (2012)	–
DX5, DX8, DX11 and DX15	Poplar	Poplar	Xylem specific	Ko et al. (2012)	–
pBdGLU1	*Brachypodium distachyon*	Maize	Endosperm specific	Coussens et al. (2012)	–
LA22CD07	Tomato	Tomato	Fruit specific	Hiwasa-Tanase et al. (2012)	–
LesAffx.6852.1.S1_at	Tomato	Tomato	Fruit specific	Hiwasa-Tanase et al. (2012)	–
Expansin promoter	*Cucumis*		Fruit specific	Unni et al. (2012)	–
Expansin promoter	Sour cherry		Fruit specific	Karaaslan and Hrazdina (2010)	–
OnTI1, OnTI2 and OnTI3 (trypsin inhibitors)	Orchid (*Oncidium* 'Gower Ramsey')	*Arabidopsis*	Flower specific	Hsu et al. (2011)	–
WM403 promoter	Watermelon	*Arabidopsis*	Nucellus (megasporangium) specific	Dwivedi et al. (2010)	Useful for developing adventive embryony
GhCesA4 promoter	Cotton	Cotton and *Arabidopsis*	Trichome and root specific	Kim et al. (2011)	Cellulose synthase subunit 4 promoter
TAPNAC promoter (At1g61110)	*Arabidopsis*	*Arabidopsis*	Anther (tapetum) specific	Alvarado et al. (2011)	–
At4g12960 promoter	*Arabidopsis*	*Brassica napus*	Outer seed coat specific	Wu et al. (2011)	–

(continued)

Table 5.1 (continued)

Promoter/cis elements	Source of identification (crop)	Tested in	Pattern of expression	References	Remarks
OsEXPB5 (expansin gene)	Rice	Rice and *Arabidopsis*	Root hair specific	Won et al. (2010)	–
HvEXPB1 (expansin gene)	Barley	Rice and *Arabidopsis*	Root hair specific	Won et al. (2010)	–
Globulin1 promoter	Maize	Maize	Seed (embryo) specific	Streatfield et al. (2010)	Extended (−1.6 Kbp) region
lycopene beta-cyclase promoter	*Solanum habrochaites*	Tomato	Flower and fruit specific	Dalal et al. (2010)	–
DIRIGENT (SHDIR16) promoter	*Saccharum* hybrid	Sugarcane, rice, maize and *Sorghum*	Stem specific	Damaj et al. (2010)	–
Omicron-methyltransferase promoter	*Saccharum* hybrid	Sugarcane, rice, maize and *Sorghum*	Stem specific	Damaj et al. (2010)	–
SBgLR promoter	Potato	Tobacco	Pollen specific	Lang et al. (2008)	*Solanum tuberosum* genomic lysine-rich
HaFAD2–1 promoter	Sunflower	*Arabidopsis*	Seed (embryo) specific	Zavallo et al. (2010)	Oleate desaturase
Promoter of Leaf Panicle 2 (Os02g40240)	Rice	Rice	Leaf specific	Thilmony et al. (2009)	–
Ppbec1 and Ppxero2	Peach	*Arabidopsis*	Cold inducible	Tittarelli et al. (2009)	–
CYP86A2 guard cell promoter	*Arabidopsis*	*Arabidopsis*	Guard cell specific	Francia et al. (2008)	Cytochrome P450 86A2
Laccase gene promoter (AtLAC15)	*Arabidopsis*	*Brassica napus*	Seed coat specific	El-Mezawy et al. (2009)	–
AGL11 gene promoter	*Arabidopsis*	*Arabidopsis*	Ovule specific	Nain et al. (2008)	–
pGC1 promoter	*Arabidopsis*	*Arabidopsis* and tobacco	Guard cell specific	Yang et al. (2008)	–

pCL and pLC,	*Arabidopsis* and potato	Potato	Cold inducible and tuber specific	Zhu et al. (2008)	Chimeric promoter combining LTRE (low-temperature-responsive element) from *Arabidopsis* cor15a promoter and the TSSR (tuber-specific and sucrose-responsive sequence) from potato class I patatin promoter
Peroxidase 1 gene promoter	Rice	Rice	ABA inducible and lateral root specific	Yu et al. (2007)	
OSIPA promoter	Rice	*Arabidopsis* and tobacco	Anther specific	Gupta et al. (2007)	Coding for proteins similar to expansins
OSIPK promoter	Rice	*Arabidopsis* and tobacco	Anther specific	Gupta et al. (2007)	Coding for proteins similar to calcium-dependent protein kinases (CDPK)
GmNARK promoter	Soybean	*Lotus japonicus*	Phloem specific	Nontachaiyapoom et al. (2007)	Nodule autoregulation receptor kinase
HARI promoter	*Lotus japonicus*	*Lotus japonicus*	Phloem specific	Nontachaiyapoom et al. (2007)	Nodule autoregulation receptor kinase

(continued)

Table 5.1 (continued)

Promoter/cis elements	Source of identification (crop)	Tested in	Pattern of expression	References	Remarks
TaPT2 promoter	Wheat	Wheat and *Arabidopsis*	P starvation and root specific	Tittarelli et al. (2007)	Putative high-affinity PO_4 transporter
NAS1 promoter	Rice and barley	Tobacco	Fe starvation specific	Ito et al. (2007)	Nicotianamine synthase
RSs1 promoter	Rice	Rice, chickpea and tobacco	Phloem specific	Saha et al. (2010)	Rice sucrose synthase1
PinB promoter	Wheat	Rice	Seed specific	Evrard et al. (2007)	Puroindoline-b gene
ALP type-B gene promoter	Wheat	Tobacco	Endosperm specific	Song et al. (2012)	Avenin-like proteins

reproductive tissues, which limits its use in cases where these structures, for example, seeds and cotton bolls, need protection (Porto et al. 2013). For use in monocotyledons, actin in rice (McElroy et al. 1990) and ubiquitin in maize (Christense et al. 1992) were identified and characterized, both being highly conserved genes across different species. Later actin from *Arabidopsis* (An et al. 1996) and ubiquitin from *Nicotiana sylvestris* (Plesse et al. 2001) were isolated for use in dicots. A list of recently characterized constitutive promoters can be found in Table 5.1.

5.2.2 Tissue-/Organ-Specific Promoters

Numerous tissue-specific promoters have been identified in plants including those from seed storage genes such as glutenin, zein, napin, legumin, tuber and root-specific glycoprotein gene and flower- and fruit-specific proteins. Cell-type specific promoters have also been identified such as those from anther, trichomes, stomata and guard cells, root hairs, phloem and cortical tissues (Table 5.1). Some of these promoters can be called as *tissue enhanced* as their expression is not always confined to a single type of tissue or organ but more in one specific tissue than others.

5.2.3 Seed-/Fruit-Specific Promoters

During seed development, seed-specific proteins are expressed in high levels, and for this reason, the seed-specific promoters became useful in genetic engineering experiments. Several seed-specific *cis* elements have been characterized (Table 5.1), especially in cereal and legume crops (Fauteux and Stromvik 2009). One of the best characterized seed-specific promoters is the French bean β-phaseolin gene (van der Geest and Hall 1996), whose strong, spatial expression has been shown in developing seeds in transgenic tobacco. Legumin is another seed-specific promoter identified from *Vicia faba* shown to be expressing well in tobacco and *Arabidopsis* (Baumlein et al. 1991). Endosperm-specific hordein promoters in barley (Forde et al. 1985), glutenin promoters in wheat (Lamacchia et al. 2001), avidin promoters in maize (Hood et al. 1997) and zein promoters in maize (Marzabal et al. 1998) are few examples of the well-studied monocot seed-specific promoters. Helianthinin promoter from sunflower (Nunberg et al. 1994), napin promoter from *Brassica napus* (Ellerstorm et al. 1996) and α-globulin promoter from cotton (Sunilkumar et al. 2002) have also been described in either one or more homologous or heterologous systems to drive embryo- and endosperm-specific expression. Apart from these seed storage specific proteins, some non-storage proteins such as sucrose-binding protein (sbp) and so-far unknown seed protein (usp) from *Vicia faba* also direct the gene expression in seeds (Zakhrov et al. 2004). Chen et al. (2007) have isolated and characterized a seed-specific promoter from the gene encoding small subunit of AGPase (ADP-glucose pyrophosphorylase) from tobacco, which

regulated expression only in seeds. Among seed-specific promoters, napin is the most commonly used promoter for transgenic expression. As early as 1991, maize oleosin with its native promoter was expressed in seed in *Brassica napus* using transgenic approach (Lee et al. 1991). Oil content in canola has been increased by seed specific expression of glycerol-3-phosphate dehydrogenase from yeast (Vigeolas et al. 2007). From tomato many fruit-specific promoters have been identified. Promoters of genes encoding ACC synthase, E8 and polygalacturonase (Deikmann and Fisher 1988; Montgomery et al. 1993) were first described from tomato and then in apple (Atkinson et al. 1998). Similarly expansin promoters from *Cucumis* and sour cherry regulate fruit tissue-specific gene expression (Unni et al. 2012; Karaaslan and Hrazdina 2010). A seed- and fruit-specific promoter of MT3-A (metallothionein-like gene) has been identified in oil palm (Kamaladini et al. 2013).

5.2.4 Tuber-Specific Promoters

Several tuber-specific promoters have been identified from tuber crops such as potatoes, yams and cassava which store starch in root organs. From potato, promoter elements of patatin family genes, B33, PAT21 and potato granule-bound starch synthase (GBSS) that induce gene expression in tubers (Jefferson et al. 1990; Visser et al. 1991), have been identified. In sweet potato, the promoters of gSPOA1, one of the genes encoding the major protein, sporamin, and gene encoding β-amylase promoters, regulate tuber-specific gene expression (Maeo et al. 2001).

5.2.5 Anther-/Pollen-Specific Promoters

Anther- and pollen-specific promoters are useful to control male sterility, thereby having an important role in hybrid seed production, an important facet of plant breeding. Numerous anther- and pollen-specific promoters have been isolated, the important ones being TA29 from tobacco, R8 from rice, A9 from *Arabidopsis*, SBgLR promoter from potato and LAT52, LAT56 and LAT59 promoters from tomato (Peremarti et al. 2010; Lang et al. 2008; Twell et al. 1991).

5.2.6 Root-Specific Promoters

Root -specific promoters are central to the development of methods/techniques to drive root-specific gene expression for nutrient and water absorption. They may also be useful for expressing defence-related genes, including those conferring insecticidal resistance and stress tolerance, e.g. salt, cold or drought tolerance. Several valuable root-specific promoters like TobRB7 from Tobacco (Yamamoto et al. 1991) have been identified and used in transgenic plant developments. Although

root-specific promoters have been isolated and functionally characterized, non-plant promoter, for instance, *rol*D promoter from *A. rhizogenes* (Elmayan and Tepfer 1995), has also found to be applicable in targeted root-specific gene expression. Promoters of EGRT2 in eucalyptus (Porto et al. 2013) and IFS (isoflavone synthase) in soybean, involved in hormone production and nodulation (Subramanian et al. 2004), also drive root-specific expression in transgenic eucalyptus and soybean roots, respectively.

5.2.7 Leaf-Specific Promoters

Leaf and other green tissues harbour chlorophyll that responds to light. Gene encoding for smaller subunit of ribulose 1,5 bisphosphate carboxylase (rbcS) harbours an intricate assembly of light-responsive and tissue-specific promoter elements for expression as revealed by the detailed analysis of pea rbcS3A (Kuhlemeier et al. 1987) and alfalfa (Potenza et al. 2004). Monocots have a different set of *cis*-acting elements as compared to dicots in rbcS promoters as found in rice and maize (Nomura et al. 2000). The promoters of genes encoding the chlorophyll a/b binding (*cab*) proteins (Anderson and Kay 1995) and other components of light-harvesting complex (*Lhc*) are characterized in tomato (Piechulla et al. 1998). They are specific to gene expression in leaves and other green tissues. Recently two novel *cis* elements directing green tissue-specific expression were isolated by Ye et al. (2012) from promoter of PDX1 gene of rice.

5.2.8 Cell-Type- and Organelle-Specific Promoters

Numerous cell-type-specific (e.g. trichome, guard cell, stomata, root hair and companion cells) and organelle-specific (e.g. plastids) plant promoters have also been isolated and successfully demonstrated for their cell-specific expression. For instance, cytochrome P450 promoters from tobacco (Clark et al. 1997) and catmint (Wang et al. 2002) confer trichome-specific gene expression. Similarly the expression of several sucrose transporters has been localized to the phloem. Notable examples are rolc promoter isolated from *Agrobacterium tumefaciens* and SUC2 promoter from *Arabidopsis* (Schmulling et al. 1989; Sauer and Stolz 1994). Wound inducible peroxidase genes from rice have been shown to possess xylem specific expression in transgenics (Ito et al. 2000). For targeting the expression to specific organelles, organelle-specific promoters are very useful. Prrn promoter of 16S rRNA (Maliga 2002), promoters of rbcL (Shiina et al. 1998), plastid polymerases (PEP) (Hajdukiewicz et al. 1997) and photosystem II promoter (psbA) (Staub and Maliga 1994) which are abundant in plastids have *cis* elements specific for driving the gene expression in these organelles.

5.2.9 Inducible Plant Promoters

Inducible promoters are those which are responsible for the expression of the associated genes in response to some physical, chemical or environmental cues and provide precise regulation of gene expression through external control. They have a broad spectrum of potential applications. For example, wound-inducible promoters are of specific interest owing to their potential role in biotic stress management. A tobacco MAP kinase genes, WIPK is known to have both wound inducible and tobacco mosaic virus specific expression (Yap et al. 2002). A wound inducible promoter has been used in expressing cry1EC in cotton to provide infection against insect bite (Kumar et al. 2009). A wound-specific promoter from coffee encoding isoflavone reductase-like protein (*CaIRL*) has been reported by Brandalise et al. (2009). Recently, the promoter sequences from −510 bp upstream to the transcriptional start site of fatty acyl-CoA reductase 6 (FAR6) gene in *Arabidopsis* were found to exhibit wound-inducible expression in the stems (Gupta et al. 2012). The wound-inducible expression got enhanced and extended throughout the mature stem, with the addition of further upstream sequences (−510 to −958, −1400 or −1456). Promoters induced by salicylic acid (SA), methyl jasmonate (MJ), jasmonic acid (JA) and ABA are important for both biotic and abiotic stress management. Promoters of ProDIR16 and ProOMT from sugarcane (Damaj et al. 2010) and FSgt-PFlt from figwort mosaic virus (Acharya et al. 2014) are some more examples for biotic stress-induced promoters identified. There are *cis*-regulatory elements specific to abiotic stress response such as promoters of rice OsNED3 and Wsi18 genes which are implicated in ABA synthesis and signalling (Bang et al. 2013; Yi et al. 2010). The most commonly used promoter for abiotic stress is Rd29A promoter from *Arabidopsis* demonstrated in wheat (Pellegrineschi et al. 2004). Artificially inducible promoters using different chemicals such as tetracycline, dexamethasone, ethanol and estradiol, singly or in combinations, have also been developed and utilized (Zuo and Chua 2000).

5.2.10 Synthetic Promoters

Synthetic promoters are sequences composed of unique combination of core, proximal and distal regulatory elements, all of which may contain specific regulatory elements. They may mimic natural promoters, but are more efficient (by keeping only the specific sequences and removing non-specific sequences from the full-length promoters, e.g. DR5 auxin promoter with tandem direct repeats of auxin-responsive element, TGTCTC) or may altogether provide novel expression profiles that do not exist in nature. They are valuable in transgenic plant development to minimize or completely escape the phenomenon of homology-induced gene silencing, by minimizing the homology with endogenous sequences and other introduced promoter sequences (Bhullar et al. 2003).

Minimal promoters (the sequences that are absolutely essential for transcription intiation; Mohan et al. 1993) and chimeric promoters are often used synthetic pro-

moters. Such promoters can be developed either by introducing known *cis* elements in novel or synthetic stretch of DNA or by replacing the functional domains of one promoter with another functionally equivalent domain from heterologous systems; latter strategy is more popularly known as 'domain swapping'. Experimental evidence, however, suggests that modifying sequences present between cis elements is a more rewarding one than domain swapping since the latter leads to fall in promoter activity whereas the former can maintain expression comparable to that of wild-type promoters (Bhullar et al. 2003). Combining specific cis-regulatory element with strong constitutive promoters is another approach for designing synthetic promoters (Rushton et al. 2002). Minimal expression cassettes and activation modules were designed from TATA box, TSS, untranslated leader and translation initiation regions of highly expressed genes in plants and the conserved sequences present in 500 bp upstream to TSS by Sawant et al. (1999 and 2001). While minimal expression cassettes were found to be sufficient to drive expression in tobacco, the use of complete expression cassettes spanning 450 bp comprising of both the minimal expression cassette and activation module was highly expressing, as high as 30-folds as compared to the native promoter, across tissues and plant species (Sawant et al. 2001). Minimal promoters containing W1, W2, GCC, JERE, S, Gst1 and D boxes, which are multiple *cis* elements present in genes that respond to pathogen attack, had been demonstrated to be active across organisms against a variety of pathogens (Rushton et al. 2002). Inducibility and strength of expression of these promoters are largely determined by spacing and motif copy number in synthetic promoters (Rushton et al. 2002), and thus appropriate designing is paramount for their functionality. Recently minimal promoters with different copy numbers of different motifs such as DRE, ABRE, MYB, MYC, G-box, as1 and rps1 site1-like were demonstrated to confer drought and salinity-mediated response in *Arabidopsis* (Hou et al. 2012).

Synthetic promoters also serve as a great resource for unravelling the role of different motifs in transcription and also to study the motif-motif and motif-protein interactions. Several synthetic promoters containing a variety of commonly found cis-acting DNA sequence motifs (G-box, OCS-element, W-box, GT1-element, GATA-box, CAAT-box, PU-element and YY1-element that are commonly present in highly expressed plant genes) were used to study the gene expression in plants by Sawant et al. (2005). These conserved sequence elements, when arranged upstream of a basal promoter, were found to function synergistically in enhancing gene expression, the complete module increasing the expression by 110-fold. Thus a *cis*-acting DNA motif could function as an activator by itself as well as a synergizing activator in the presence of other homologous as well as heterologous motifs in the neighbourhood. The results suggested a hierarchical assembly of several motif-binding factors, leading to the stabilization of the transcriptional complex formed on the TATA box.

The role of two *cis* motifs, namely, ACGT and GT, both of which regulate pathogen defence, was unravelled by the use of minimal promoters (Mehrotra et al. 2005; Mehrotra and Panwar 2009). A single ACGT motif enhanced promoter activity by nearly twofold, when placed 100 nucleotides upstream to TATA box, while two ACGT motifs separated by five nucleotides enhanced promoter activity by sixfold, when placed 50 nucleotides upstream to minimal promoter. A recent study indicated

that two copies of ACGT when separated by five nucleotides allowed promoter activation by salicylic acid, but when separated by 25 nucleotides, the promoter activity was induced only by abscisic acid but not salicylic acid. Furthermore, single GT elements enhanced activity by nearly twofold when placed 50 nucleotides or 100 nucleotides upstream to promoter, but when two GT elements separated by 5, 10 or 25 nucleotides were placed, the promoter activity plummeted. Hence, it was proved that a second copy of GT negatively regulates the activity of promoter, when placed in close proximity to minimal promoter (Mehrotra and Mehrotra 2010).

Bidirectional promoters which have transcription activation modules in both the orientations are known in literature, for example, CaMV35S promoter, *mas* (mannopine synthase from *Agrobacterium tumefaciens*) promoter, oleosin promoters from *Brassica napus* and *CaTin1* and *CaTin1–2* promoters from *Capsicum annuum*. Bidirectional promoters have the potential advantage of simultaneously expressing two genes, thus halving the number of promoters needed for expressing transgenes. Such promoters are particularly relevant for co-expressing pairs of gene coding for subunits of antibodies, proteins which are needed in equimolar quantities and for expressing a pair of genes of a synthetic pathway (Pratibha et al. 2013; Sharma et al. 2015). A construct with the commonly found conserved elements mentioned earlier along with motifs for 14 different elicitors was used to design a synthetic bidirectional promoter, and their expression was studied through transient and stable transformation assay (Chaturvedi et al. 2006). The transcription activation module responded to elicitors like salicylic acid, NaCl and IAA in the forward as well as reverse directions showing that constitutive and chemically inducible bidirectional promoters can be deployed for predictable and simultaneous regulation of two genes for genetic engineering in plants.

A basic as well as a challenging requirement of transgene technology is high level of transgene expression. In addition to strong promoters such as CaMV35S and RbcS, 5′UTRs are also known to enhance transgene expression. In this regard, a 28 bp long synthetic 5′UTR, named synJ, has been demonstrated to enhance gene expression by 10- to 50-fold in tobacco and cotton, as compared to the reporter genes under CaMV35S. synJ can work equally well under weak promoters such as nos (Kanoria and Burman 2012).

Stringently controlled transgene expression modules that are on target both spatially and temporally can be designed based on two-component systems. The component one generally consists of a minimal promoter and a target promoter with specific TF-responsive motifs which is inactive when present alone. Component two is a chemically inducible TF driven by a constitutive promoter that specifically recognizes component one. Thus the component one would work only in the presence of the functional component two (Padidam 2003). A two-component system without involving chemicals for initiation of transcription is reported by Chaturvedi et al. (2007). The component one called as expression module has highly expressing TATA-dependent seed promoter in which the TATA motif in the core promoter is mutated. The component two, called as regulatory module, has a mutated TF that can recognize the mutated TATA motif in the core promoter of expression module. This system has been demonstrated to be expressing in tightly seed-specific manner

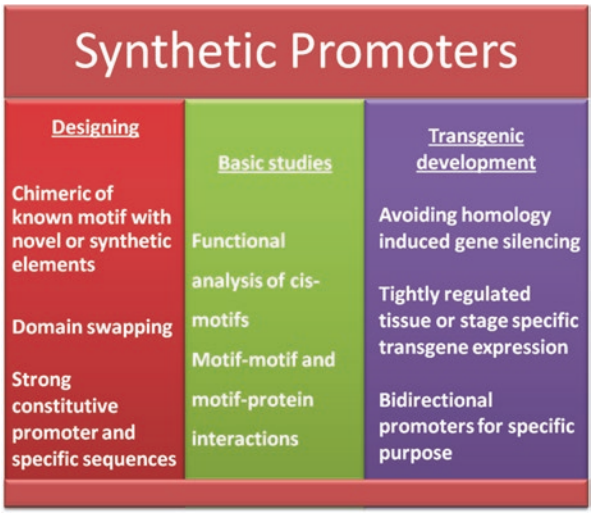
Synthetic Promoters
Designing
Chimeric of known motif with novel or synthetic elements
Domain swapping
Strong constitutive promoter and specific sequences
Basic studies
Functional analysis of cis-motifs
Motif-motif and motif-protein interactions
Transgenic development
Avoiding homology induced gene silencing
Tightly regulated tissue or stage specific transgene expression
Bidirectional promoters for specific purpose

Fig. 5.2 Synthetic promoters: design and applications

in tobacco. Thus, by modifying the structure of a TF and coupling it with a tissue-/stage-specific promoter in a two-component system, transgene expression can be tightly regulated at will.

5.3 Isolation and Characterization of Promoters

Understanding gene regulation is one of the most exciting topics in molecular biology. To learn how the interplay among thousands of genes leads to the existence of a complex eukaryotic organism, identification and understanding of promoters is a prerequisite. The practical importance of understanding the molecular switches is the potential they offer to influence and direct plant metabolism as tuned to the external environment for their better survival and reproduction and to achieve compartmentalized production of valuable pharmaceutical and nutraceutical products. The approaches to identify promoters can be broadly classified into two major categories: bioinformatic and functional genomic approaches (Fig. 5.2).

5.3.1 Bioinformatic Approaches

Computational prediction of promoters from nucleotide sequence is one of the attractive approaches for promoter identification. In early days of bioinformatic promoter search, promoter prediction focused on identifying the promoter of protein-coding genes (Fickett and Hatzigeorgiou 1997), but now it is being performed across the genome as it has been shown that transcription occurs all over the genome (Carninci

et al. 2006; Frith et al. 2008; Sandelin et al. 2007). Many promoter prediction programmes (PPPs) developed for this purpose are freely available and have been successfully used for eukaryotic promoter prediction (Helden 2003; Kumari and Ware 2013; Table 5.2). These algorithms search promoters, either based on known motifs from the databases containing experimentally validated motifs or based on phylogenetic footprinting, using multiple alignment tools and different statistical approaches.

Searching for promoter sequence motifs in a genome, computationally, is very different from searching for a gene, primarily because a gene has well-defined structural features whereas a promoter does not; the latter is rather defined functionally than structurally and hence more challenging. *In silico* approaches to search for promoters can be primarily divided into two types, namely, alignment and enumerative methods. Some algorithms do employ both the strategies in their search for promoters esp. when they do *de novo* promoter search rather than depending solely upon previous information available. Such algorithms are known to be more efficient. Algorithms that use prior information to identify promoter motifs compute position frequency matrices. Position frequency refers to the number of times a nucleotide has occurred at a given position. Promoters are typically searched in either intergenic regions or in proximal promoter regions of 500 bp–1 kb upstream to start codon. One of the important features of proximal regulatory regions is that they are least populated with transposable elements.

Alignment methods primarily carry out multiple local alignments (MLA) of all sequences of interest (usually co-regulated genes) using appropriate tools. MLA in itself is very challenging since the sequences are gathered from multiple sources. Some MLA tools do sequence alignment one by one and in the process optimize the information content by computing weight matrices for each alignment (Hertz and Stormo 1999). Recent algorithms use different optimization procedures that do direct multiple alignments and employ probabilistic models such as expectation maximization model to find statistically significant motifs. Enumerative are also called as exhaustive methods since they examine all oligomers of a fixed length and identify those sequences that are over-represented as compared to the overall background distribution of the rest of the data set (van Helden et al. 1998; Brazma et al. 1998). Enumerative methods employ different statistical means to identify the over-represented motifs. One of them considers normal distribution and estimates p-values associated with z-scores of the distribution and identified promoters successfully in higher eukaryotes. Enumerative methods are more popular since the arrival of whole-genome sequencing data.

Promoters can be predicted either from genome sequence data or from expression data. However, mere identification of *cis* elements and annotating them as a particular type of promoter is not sufficient to predict transcriptional product of a transgene since a *cis* element active under the control of one promoter may not transcribe desired protein with another promoter (Tiwari et al. 2003). Hence, it is of utmost importance to annotate all the sequences in a genome and represent introns, exons, trans-acting factors and *cis*-regulatory elements in promoters with the help of regulatory networks and logic functions.

Table 5.2 Computational biology tools and database for promoter prediction and identification

Programme/database/tool	Description
AlignACE	Motif-finding algorithm
AGRIS	Currently contains two databases, AtcisDB (*Arabidopsis thaliana* cis-regulatory database) and AtTFDB (*Arabidopsis thaliana* transcription factor database)
AthaMap	A genome-wide map of putative transcription factor binding sites in *Arabidopsis thaliana*
AtProbe	The *Arabidopsis thaliana* promoter-binding element database, an aid to find binding elements and check data against the primary literature
CpG promoter	Promoter mapping using CpG islands
Core promoter	To predict putative TSS
DBTSS	Database of TSS
Dragon Promoter Finder	An advanced system for promoter recognition in vertebrates
EPD	An annotated nonredundant collection of eukaryotic POL II promoters
FirstEF	A 5′ terminal exon and promoter prediction programme
Gibbs sampler	Alignment method-based promoter prediction tool
McPromoter V3	A statistical tool for the prediction of transcription start sites
MEME	Alignment via expectation maximization for promoter prediction
Motif Explorer	Motif and promoter visualization
Neural Network Promoter Prediction	Search-by-signal, time delay neural network
Paint	Promoter analysis and interaction network toolset
PLACE	See text for details
PlantCARE	See text for details
PlantPAN	See text for details
PlantProm DB	See text for details
PlantPromoterDB	See text for details
ProGA	Eukaryotic promoters recognition by genetic algorithm
PromoterInspector	Search-by-content, class-specific oligomers
PromoSer	Promoter extraction service
Promoter 2.0 Prediction Server	Predicts TSS of vertebrate PolII promoters in DNA sequences
Promoter Prediction	Scans vertebrate nucleotide sequences for putative promoters
Promoter Prediction - U. Ohler	Exact localization of eukaryotic RNA polymerase II TSS
PromoterWise	BioPerl module for promoter identification
Proscan	Scores homologies with putative eukaryotic Pol II promoter sequences
Regulatory Region Analysis	First searches in a nucleotide sequence for putative promoter regions and subsequently scans them for possible transcription factor binding sites
RSA Tools	Yeast and microbial exhaustive

(continued)

Table 5.2 (continued)

Programme/database/tool	Description
TRANSFAC	See text for details
Tfsitescan	Intended for promoter sequence analysis
TSSG	Recognition of human PolII promoter region and TSS
TSSG/W	Predicts potential TSS by linear discriminant function

In the past two decades, annotated genome sequences of many plants became publicly available, and this information is being used for functional analysis of the sequences. Ettwiller et al. (2003) had predicted *cis* elements in *Saccharomyces cerevisiae* genome using functional networks, mainly protein–protein interactions and metabolic networks. They presented them as patterns using pattern discovery tool such as Teiresias and represented them as motifs. The interest in promoter analysis received a great boost with the arrival of microarray gene expression data. Research till then had focused on the detection of single motifs (representing transcription factor binding sites) common to the promoter sequences of putatively co-regulated genes (Ohler and Niemann 2001). Although this problem might seem simple at first, it is very complex, because of three main points. First, the motif is of unknown size and might not be well conserved. Second, the sequences used to search for the motif do not necessarily represent the complete promoter. Third, genes with promoters to be analysed in many cases are grouped together by a clustering algorithm. As this algorithm might be error-prone, the genes are not necessarily all co-regulated *in vivo*. Because of these reasons, the search has been focused on model organisms with smaller genome sizes such as *S. cerevisiae*. The arrival of expression arrays yielded enormous amounts of data linking genes, *via* their cDNA sequences, to gene expression patterns. This permits the characterization of gene expression in normal and treated tissues. Expression array data can be analysed with respect to the underlying protein sequences, which facilitate precise determination of when and where certain groups of genes are expressed. Once the details of expression of certain genes, like tissue specificity and stage of expression, are made available, the promoter elements of such genes can be obtained from nucleotide sequence by methods such as exon mapping. Also there can be approaches that can provide powerful alternatives for elucidating the functional features of genes with no detectable sequence similarity, by linking them to other genes on the basis of their common promoter structures (Werner 2000). Following the deluge of expression data, many algorithms for promoter prediction based on expression databases were developed (Hehl and Wingender 2001). An algorithm called 'PromoterInspector' to locate eukaryotic polymerase II promoter regions in large genomic sequences with a high degree of specificity was developed by Scherf et al. (2000). Tools like neural network promoter prediction (Reese 2001) and PromoSer (Halees et al. 2003) have also been most widely used.

The pairing of genome sequence databases to genome-wide transcriptomic analysis has now become possible and would be quite useful for prediction of numerous *cis* elements with diverse functionality (Hernandez-Garcia and Finer 2014). Such

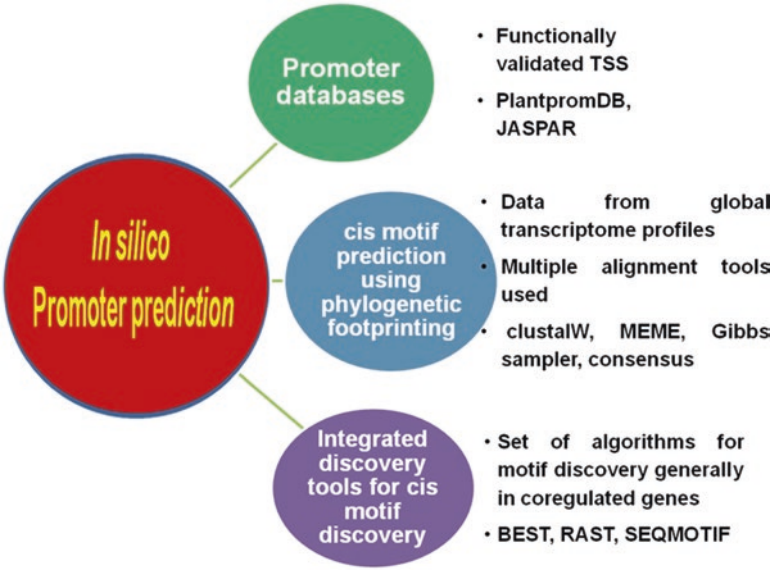
In silico
Promoter prediction
Promoter databases
• Functionally validated TSS
• PlantpromDB, JASPAR
cis motif prediction using phylogenetic footprinting
• Data from global transcriptome profiles
• Multiple alignment tools used
• clustalW, MEME, Gibbs sampler, consensus
Integrated discovery tools for cis motif discovery
• Set of algorithms for motif discovery generally in coregulated genes
• BEST, RAST, SEQMOTIF

Fig. 5.3 Schematic representation of *in silico* promoter predication strategy and tools

genome-wide analysis has been done in *Arabidopsis*, rice and soybean to identify abiotic stress-responsive elements (Ibraheem et al. 2010; Maruyama et al. 2012). These studies provide a better understanding of global gene regulation at the organism level. The only major drawback is that they identify a huge number of putative elements. Core promoter elementsacross the entire genome from both monocots and dicots have been computataionally predicted recently by Kumar and Ware (2013). Many new promoter prediction programmes (PPPs) have emerged in the last decade. In most cases, however, only a small portion of the genome is used to evaluate the programme, which is not a realistic setting for whole-genome annotation projects (Abeel et al. 2008). Moreover, there is quite a mismatch between functionality and the presence of short sequence motifs since the former depends on physical position (location) and accessibility and presence of other accompanying elements that would enable protein binding (Hardison and Taylor 2012). For instance, in mammalian genomes, eight million GATA1 sequence motifs are present, whereas only 15,000 of them are shown to be functional in mouse, as revealed by histone modifications and mRNA transcription (Cheng et al. 2009; Zhang et al. 2009). Thus, the comparative analysis *in silico* is only the initial step in identification of promoter which needs to be followed up by detailed functional *in vivo* analysis (Fig. 5.3).

Certain regulatory sequence analysis tools are freely available that allow retrieval of sequences in genomes, pattern discovery, pattern matching with putative TF sites and TF-binding sites. Till date there are six main databases that identify transcription factor binding sites in upstream *cis*-regulatory elements in plant promoters. First is PLACE (plant cis-acting regulatory DNA elements) that compiles all the

motifs in plants that have been originally reported, their variations in other genes or in other plant species reported later, along with a brief description of each motif (Higo et al. 1999). Second one is PlantCARE (plant cis-acting regulatory element) that compiles information about transcription factor sites, motif sequence, function, species, cell type and genes and represents *cis* elements by positional matrices, consensus sequences and individual sites on particular sequences (Lescot et al. 2002). This database also allows identification of new *cis* elements through *in silico* analysis. Third database, PlantProm DB (plant promoter database), is an annotated nonredundant collection of proximal promoter sequences for RNA polymerase II with experimentally determined TSS from various plant species. Currently, it contains 578 unrelated entries including 151, 396 and 31 promoters with experimentally verified TSS from monocot, dicot and other plants, respectively. Since this DB contains only the published promoter sequences with TSS(s) determined by direct experimental approaches, it serves as the most accurate source for development of computational promoter prediction tools. Moreover, 3503 and 4220 promoters with TSS predicted by mapping full-length cDNAs on genomic sequences from *Arabidopsis* and rice were added to new release of this DB. Totally, 8301 entries of plant promoters are available in current release of PlantProm DB. Fourth one is PlantPAN (plant promoter analysis navigator) which, apart from TF-binding site analysis of the *cis* elements, also allows analysis of tandem repeats, CpNpG islands and miRNA targets present in the query sequence (Chang et al. 2008). The fifth one, PlantPromoterDB, has information on several plant species, currently encompassing TSS information of *Arabidopsis thaliana*, *Populus trichocarpa*, *Oryza sativa*, *Chlamydomonas reinhardtii* and *Physcomitrella patens*. It can identify *cis* motifs, relating to the TSS predicted from the query sequence. TRANSFAC (transcriptional regulation, from patterns to profiles), the sixth one, compiles eukaryotic transcription factors, their target genes and regulatory binding sites (Matys et al. 2003). It is the only database that provides structural and functional information of transcription factors. However, this is a commercial site. There is another database called PromoSer which can identify different cis acting and regulatory elelments from a given sequence (Halees et al. 2003). There are also other smaller databases pertaining only to specific species such as *Arabidopsis* or *Homo sapiens* or both (Table 5.2).

5.3.2 Functional Genomic Approaches

Although bioinformatic approaches use all the available information such as co-expression or co-regulation networks, phylogenetic footprinting information (orthologous expression across species) and the sequence, distance and orientation of the known motifs from the TSS, they are not foolproof. Hence, functional genomic approaches which are more reliable have to be employed simultaneously. Traditional approach for identification of promoter was to clone the adjoining DNA sequence from a genomic library using the transcribed part as a probe. This approach has led to the identification of several promoters in the past few decades. However,

this is a laborious and demanding approach. The recent developments in functional genomics have given rise to several methods for gene expression analysis such as suppression subtractive hybridization (SSH), serial analysis of gene expression (SAGE), massive parallel signature sequencing (MPSS), microarray, differential display technique and transcriptome sequencing from different tissues/stages/conditions that can be used for identification of transcripts. Once the transcripts are identified, the regulatory regions of those genes can subsequently be cloned and characterized. However, most of these approaches are technically demanding and hardly detect genes that are expressed at low levels. Moreover, they pose difficulty in identification of promoter element of an individual gene belonging to a gene family. Validation of huge number of *cis* elements identified from microarray or transcriptome sequence data would also be a very huge task. Recently, a new high-throughput methodology called 'self-transcribing active regulatory region sequencing' (STARR-seq) has been used in *Drosophila* for this purpose, which allows quantification of enhancer activity of millions of candidate enhancer containing regions (Arnold et al. 2013). This technology uses screening vectors that contain small pieces of DNA isolated from sheared genomic DNA and cloned downstream of a minimal promoter so that potential enhancers alone would be self-transcribed. Reporter libraries are then transfected into candidate cells, and through high-throughput paired end sequencing of the transcripts, potential enhancers are identified. This technology could be a powerful tool to identify promoter and enhancer motifs in plants (Hernandez-Garcia and Finer 2014).

Insertional mutagenesis (IM) is the most direct approach for discovering or validating gene function and hence exploited more frequently in isolating plant genes and their regulatory elements. It is a means of analysing the function of genes through their disruption by foreign DNA followed by observation of the consequence/effect on phenotype. IM for promoter isolation is also called as promoter tagging or promoter trapping which involves delivery of promoterless reporter genes into plant genomes and assay of reporter gene activity in the transgenics. Insertions are generally mediated either by transposon or T-DNA. This method was first used in *Arabidopsis* and later in rice and other crops successfully. In light of the ongoing accumulation of more and more DNA sequences of unknown function, insertional mutagenesis offers the direct means for determining the function of newly identified sequences. As compared to chemical, physical or any other random mutagenesis, IM-based strategies are preferred because the insertion sites are easy to follow and flanking sequence tags (FSTs) can be created for all the insertions in the library which allows one to follow up the entire genome (Wang et al. 2013). The availability of genome sequence information in many important crops complements this approach by enabling cataloguing of the insertions across the genome. The knockout mutations thus created may not always be effective in functional analysis since, in case of redundant genes, loss of function may not lead to an obvious morphological variation. Some genes are expressed or needed by the plant system only under specific environmental cues or conditions such as biotic or abiotic stresses and hence their identification would require specific screening procedures., Disruption in critical genes required for plant growth and development would lead to lethality and hence their recovery and identification is not possible (Wang et al. 2013).

The strategy begins with broad insertional mutagenesis of the genome through T-DNA or transposons followed by identification of individuals carrying insertions in the gene of interest and their phenotypic and functional analysis (Ballinger and Benzer 1989; Kaiser and Goodwin 1990). In principle, the insertion of T-DNA and transposable elements into coding or regulatory sequences of a gene can often disrupt gene function (Feldmann 1991; Martienssen 1998a), and such insertions are identified by polymerase chain reaction (PCR) using a combination of a gene-specific primer and a primer complementary to the T-DNA or transposon border sequences. The reporter gene in a T-DNA or transposon literally traps or tags an element conferring the expected expression behaviour.

Transformation requires an efficient selection system in order to distinguish between transformed and non-transformed plant cells. The reporter system helps monitor tissues to assess the success of a specific construct/protocol in transformation. Therefore, selectable and reporter genes form critical components of advanced plant promoter research (Schrott 1995). The genes specifying β-glucuronidase (gus), luciferase (luc) (Jefferson et al. 1987; Ow et al. (1986) and green florescent protein (gfp) (Chiu et al. 1996) are the most widely used reporter genes in insertional mutagenesis research.

In *Arabidopsis*, most reverse genetics screens have used T-DNA insertional mutagenesis. This process requires the use of large populations so that only a few inserts per plant would result in genome saturation. A large number of insertion lines have been generated in *Arabidopsis*, and reverse genetics screening was successfully applied to identify insertions in targeted genes (Krysan et al. 1996; McKinney et al. 1995). Benefiting from experience accumulated over the last decade in the model dicotyledonous species of *Arabidopsis*, large insertion line libraries have been generated in rice, the model plant for cereals and grasses. Different insertion mutagens encompassing T-DNA, the genetically engineered maize transposable Ac/Ds system and the endogenous ty1-copia retroelement Tos17 have been used for functional genomic studies.

5.3.2.1 T-DNA-Based Traps

T-DNA-based traps for IM are more recent as compared to transposon-mediated methods. *Agrobacterium*-mediated transformation technique has evolved significantly over the years making T-DNA a viable method for genome-wide IM. T-DNAs as the insertional mutagen offer a major advantage in terms of stable integrations across multiple generations, as opposed to transposons which transpose subsequent to integration within the genome, making their characterization, in terms of both phenotyping and molecular, difficult (Martienssen 1998b; Wisman et al. 1998). As mentioned earlier in this chapter, IM methods are attractive because of their amenability to create a FST library of insertions. Many PCR-based methods have been developed that allow one to identify FSTs of T-DNA insertion in a mutant population and subsequently isolate plants that have mutations in the gene(s) of interest (McKinney et al.1995; Krysan et al. 1996). T-DNA insertion constructs can be

suitably modified to isolate different elements of eukaryotic genes. Such constructs have been used in *Arabidopsis* as gene trap (Babiychuk et al. 1997), promoter trap (Lindsey et al. 1993) and in rice as activation tagging (Weigel et al. 1996) for functional genomics.

Besides the general issue of IM products coming under the purview of transgenic regulations, the T-DNA-based insertional mutants have the specific issue of nature of integration of the transgene. Integration of the T-DNA results, more often than not, in either tandem direct or inverted repeats or deletions in one or more borders, making the integration complex. Subsequent molecular analyses become difficult owing to such rearrangements which in turn adversely affect the success of large-scale strategies such as FST databases. Moreover, complex and multiple insertions are likely to lead to artefact and unreliable patterns of reporter gene expression especially with entrapment vectors such as gene and enhancer traps. This is usually not an issue in case of knockout mutants. Finally, T-DNA approach is not feasible in those plant species where transformation methods are slow or labour intensive or in species which are recalcitrant to *Agrobacterium*-mediated transformation. However with increasing number of species becoming amenable to transformation, this may not be an issue. Further, *in planta* transformation methods are now available which can overcome the need for *Agrobacterium*-mediated transformation and regeneration, avoiding the cumbersome tissue culture procedure and making most of the species amenable for transformation.

5.3.2.2 Promoter Trap

A promoterless reporter gene with a selectable marker is placed between the T-DNA borders and used for transformation in a promoter trap. Insertion of the promoterless reporter not only disrupts normal gene function but also activates expression of the reporter gene, when the insertion occurs in an exon of an actively transcribing gene. Promoter traps allow an ingenious way of identifying promoters that are specific to expression in particular tissue/stage. For instance, in the case of GUS as the reporter gene, detection of GUS activity in a particular organ or cell or a particular developmental stage will lead to identification of promoter sequences responsible for gene expression at this tissue/organ and time. Such a promoter trap-based mutant resource in *Arabidopsis thaliana* has been generated by our group at National Research Centre on Plant Biotechnology in India (Radahmony et al. 2005; Resminath et al. 2005; Srinivasan and Saha 2010). From this resource, a LOJ (lateral organ junction)-specific promoter was initially reported by Prasad et al. (2005). Further characterization of this promoter, through deletion analysis, revealed anther- and seed-specific elements which were masked by the LOJ-specific elements (Saha et al. 2007a and 2010). Pentatricopeptide motifs were also identified in this promoter region (Saha et al. 2007b). Later, a novel enhancer element was identified from the upstream sequences of the same LOJ gene (Saha et al. 2011). This effort also led to development of a simple procedure for identification of enhancer elements (Saha et al. 2012). Using this mutant resource, independent transgenic lines with anther specific (Thakre et al. 2006) and root specific promoters (Sivanandan

et al. 2005) were identified. From the upstream region of S-Adenosyl homocysteine hydrolase gene of a mutant, yet another anther and seed specific promoter element was identified (Sujatha et al. 2009).

A promoter with wound-inducible and stem-specific expression was isolated from the same resource (Gupta et al. 2012). Recently, a bidirectional cryptic promoter with anther-specific expression has also been isolated and characterized (Pratibha et al. 2013).This also led to the identification of a mitochondrial gene, Coproporphyrinogen which is essential for gametophyte development in Arabidopsis (Pratibha et al. 2017). Another bidirectional promoter specific to embryo sac was identified by Sharma et al (2015). Cryptic promoters have also been identified from this resource (Sivanandan et al. 2005; Parvathy et al. 2016).

5.3.2.3 Enhancer Trap

An enhancer trap construct carries a reporter gene fused to a weak or basal or minimal promoter, which on its own is insufficient to drive the detectable expression of the reporter gene. It, however, can respond to transcriptional enhancers that lie in vicinity of the construct. In enhancer trap, a reporter gene cassette containing a minimal promoter close to the end of the insertion element can be *cis* activated, and its expression is detected when inserted close to a transcriptional enhancer.

Enhancer traps have the same advantage of promoter trap in identifying elements that are specific to expression in particular tissue/stage. A disadvantage, however, is that it may not be easy to identify and locate the enhancer element identified through enhancer trap, since the enhancer element could be on either side of the T-DNA and could also be at a far off distance.

5.3.2.4 Transposon as an Insertion Vehicle for Gene Traps

Depending upon the properties of the transposon such as the mechanism and control of transposition, and those of the host plant system such as transformation effectiveness, many strategies have been developed for genome-wide transposon-mediated IM. In *Arabidopsis* and rice, these strategies have been successfully employed subsequent to which databases for screening for the knockout of a gene of interest have become a reality.

The most common transposable element used in IM is Ac/Ds of maize. Ac is activator and has transposase activity intact and hence can jump at will. Ds is typically Ac devoid of transposase activity and hence cannot jump on its own. It is dependent on Ac for transposition. Ac/Ds was found to be active even in a heterologous system, tobacco *(Nicotiana tabacum)* (Baker et al. 1987). Subsequent to this finding, the maize Ac/Ds elements have been exploited in tagging studies in a number of species including *Arabidopsis*, rice, tomato, carrot, potato, etc.

In transposon-based IM, single- and two-component-based strategies are available. In one component system, an autonomous element which can transpose freely

is transformed into a plant and propagated for five to seven generations by single seed descent. Though this system is technically less demanding and easier to adopt, genome stability remains a serious concern, since the active component can jump in and excise out at its will, making molecular and phenotype analyses difficult and unreliable. In a two-component system, the autonomous and nonautonomous components of a transposon are independently used for generating transgenic lines. One plant is transformed with a construct harbouring autonomous element (Ac or Spm/En) that provides the source for transposase. Another plant is transformed with the nonautonomous element (Ds or dSpm/I), which is dependent upon the former transgenic for its transposition. The constructs with nonautonomous elements are engineered to have selectable markers such as genes for antibiotic resistance or herbicide resistance (positive selection) to select for the presence of transposed elements. Similarly, the autonomous element constructs have negative selection markers which can be later used for selecting against them. Plants homozygous for autonomous element are crossed with plants homozygous for nonautonomous element to facilitate transposition. Progeny lines with stable and single insertions can be obtained by selecting for antibiotics or herbicide resistance and selecting against transposase relevant markers from the segregating progeny.

Tissier et al. (1999) designed a modified Spm/En system, in which the Spm transposase and the nonautonomous dSpm elements are contained within the same T-DNA construct. This system, thus, eliminates the need for crossing and also reduces the number of progeny required for selection, as the negative selection is applied against only a single locus. However in this case, the maintenance of starter lines is problematic, as the dSpm elements will continually transpose in the presence of the Spm transposase. Using a relatively large number of independent starter lines obtained through transformation may tackle this issue.

The major advantage of the two-component strategy is that it permits near-saturation mutagenesis of any genome with a relatively small number of plants. The insertions generated by transposons are generally single and intact (no rearrangements) and hence are more amenable for molecular analysis. Thus, the complications arising due to artefact and unreliable expression of complex integrations, usually found in T-DNA-based entrapment vectors, are absent here. Reversion phenotypes and incomplete reversions resulting in weaker phenotypes are common with transposon-based IM mutants. This feature provides an attractive means for validation of the mutant gene function. Since production of a transposon IM population involves the jumping nature of transposon, it is sufficient if only a few independent transgenic events are generated. Hence this can be useful even in species with poor transformation efficiency. In case of elements such as Ac-Ds which have propensity towards transposing to genetically linked sites, targeted saturation of a genomic region with mutagenesis is possible (Ramachandran and Sundaresan 2001).

Disadvantages may arise from the continuous nature of the transposition events, even though it is restricted to one round of (F1) meiosis and just one generation of mitosis. The presence of footprints in genes due to imprecise excision will also be detected by PCR which will lead to mutations that are not tagged (Ramachandran and Sundaresan 2001).

5.3.2.4.1 Mutant Resources

Rice and *Arabidopsis* are considered as model organisms for monocots and dicots, respectively. As the genomes of both the plants are sequenced and annotation carried out, the related database of these plants are contributing significantly to the functional genomics. Since all the tools of functional genomics are based on the analyses in phenotypic variations among the wild types and their mutants, the generations and collection of mutant resources form the basis of the technological aspects of functional genomics. With regard to the model crops described above, a large number of mutants have been generated in the past few decades employing different means of mutagenesis such as maize two-element Ac/Ds system and T-DNA insertion mutagenesis.

Several gene entrapment and activation-tagged lines of *Arabidopsis* have been developed and available online (Table 5.3). In rice, gene trap and enhancer trap constructs have been designed to facilitate the analysis of genes based on their expression patterns. T-DNA activation tagging populations have been developed using vectors with CMV 35S enhancer multimers, the inserts characterized by FSTs and phenotype data for forward and reverse genetics screens. The mutants can be used for functional genomic studies by researchers across the world after obtaining from the respective institutes/agencies. Since the insertional mutants are developed through transgenic approaches, appropriate permissions need to be obtained before procuring them.

5.4 Characterization of Regulatory Regions Using T-DNA Insertional Mutagenesis

Insertional mutagenesis is an alternative means of disrupting gene function and provides a rapid way to clone a mutated gene. The insertion of foreign DNA into the gene of interest not only disrupts the expression of the gene but also acts as a marker for subsequent identification of the mutation. The insertion is either through transposable elements (Sundaresan et al. 1995; Martienssen 1998a, b) or by T-DNA of *Agrobacterium tumefaciens* (Azpiroz-Leehan and Feldmann, 1997). An advantage of using T-DNA as the insertional mutagen as compared to transposons is that the flanking sequences of the mutant gene can be used to isolate the mutant gene or entrapped elements such as promoters/enhancers/genes/polyA tails (Srinivasan and Saha 2010). Also T-DNA insertions do not transpose subsequent to insertion and are chemically and physically stable through multiple generations. Different strategies are available to isolate the mutant gene or entrapped elements.

5.4.1 Plasmid Rescue

This is one of the conventional approaches used for isolating and cloning the promoter elements. The DNA fragments flanking the T-DNA are identified from the clones of the genomic library of the mutant plant and used as a probe to isolate the

Table 5.3 Mutant resources for *Arabidopsis* and rice

Crop	Database	References
Arabidopsis	T-DNA Express|AtTome	http://signal.salk.edu/Source/AtTOME_Data_Source.html
	AGRIKOLA: systematic RNAi knockouts in *Arabidopsis*	http://www.agrikola.org/index.php?o=/agrikola/main
	Biological Research Center (Hungary)	http://web.szbk.u-szeged.hu/arabidop/mappingoftdnalines.htm
	CSHL	http://genetrap.cshl.org/
	GABI-Kat	http://www.gabi-kat.de/
	FLAGdb	http://urgv.evry.inra.fr/projects/FLAGdb++/HTML/index.shtml
	RIKEN: Mutant Line Database	http://rarge.psc.riken.jp/line/
	RIKEN: Activation Tagging Line Database	http://amber.gsc.riken.jp/act/top.php
Rice	Transposon Insertion Mutant Database	http://www-plb.ucdavis.edu/Labs/sundar/Rice_Genomics.htm
	Rice Mutant Database - National Center for Plant Gene Research	http://rmd.ncpgr.cn
	Rice Tos17 Insertion Mutant Database	http://tos.nias.affrc.go.jp/
	Taiwan Rice Insert Mutant Database (TRIM)	http://trim.sinica.edu.tw/
	Oryza Tag Line	http://urgi.versailles.inra.fr/OryzaTagLine
	KRDD: Korean Rice Ds-tagging lines Database for Rice	http://www.niab.go.kr/RDS
	Shanghai T-DNA Insertion Population	http://ship.plantsignal.cn/home.do
	TRIM (Taiwan Rice Insertional Mutants Database)	http://trim.sinica.edu.tw/

genomic sequence from wild-type plants. Though this is a tedious approach, it suits when tandemly repeated copies of T-DNA are present at the same location (Marks and Feldmann 1989).

In this approach, the construct for mutagenesis carries a bacterial antibiotic resistance gene and a bacterial origin of replication *(ori)*. The genomic DNA of mutant plant is digested by an appropriate restriction enzyme followed by ligation to circularize all the fragments (intramolecular ligation), which are later transformed into

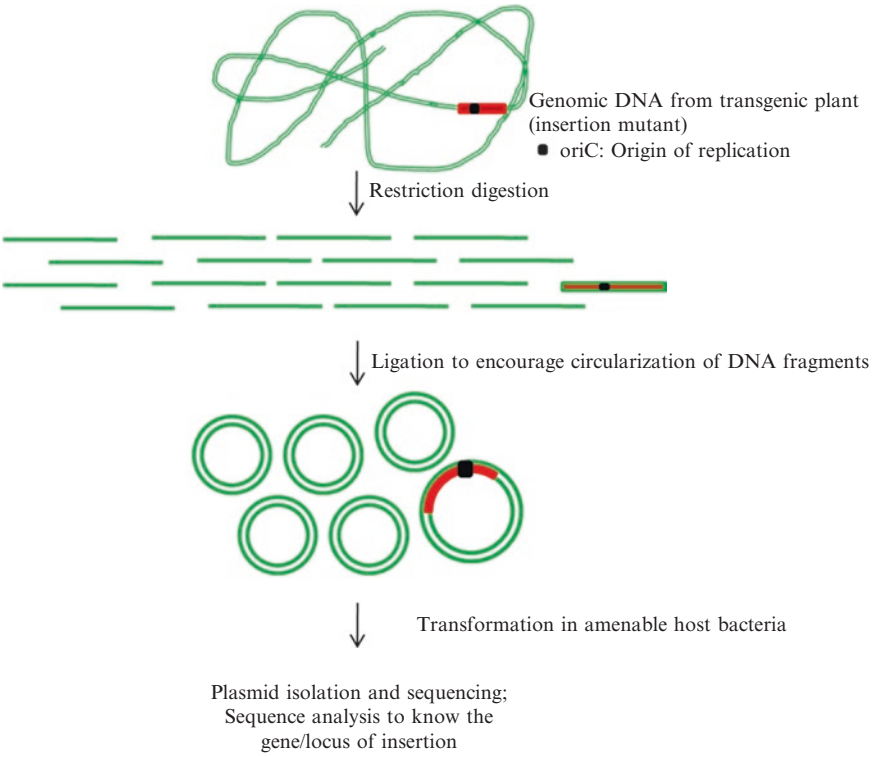
Genomic DNA from transgenic plant
(insertion mutant)
oriC: Origin of replication
Restriction digestion
Ligation to encourage circularization of DNA fragments
Transformation in amenable host bacteria
Plasmid isolation and sequencing;
Sequence analysis to know the
gene/locus of insertion

Fig. 5.4 Schematic representation of plasmid rescue for insertion mutant analysis

the host, *E.coli.* The principle of plasmid rescue is that only circular DNA fragments with intact *ori* sequence and the antibiotic resistance gene can survive in *E.coli* grown on medium supplemented with antibiotic (Yanofsky et al. 1990). The plasmids isolated and recovered from the host are analysed by PCR and sequenced for the presence of T-DNA and the flanking plant DNA sequences (Fig. 5.4). Plasmid rescue or an adapter PCR method was used to identify 1172 independent genomic loci of T-DNA integration sites in the activation tagging lines of *Arabidopsis* (Ichikawa et al. 2003).

5.4.2 Adapter Ligation-Mediated PCR

Unlike plasmid rescue, this method does not require any additional special features in the construct designed for mutagenesis. The procedure consists of three major steps: digestion of genomic DNA with restriction enzyme followed by ligation of the restriction enzyme specific to the adapter to the restriction fragments; a PCR amplification of the T-DNA/genomic DNA junction with primers specific to the

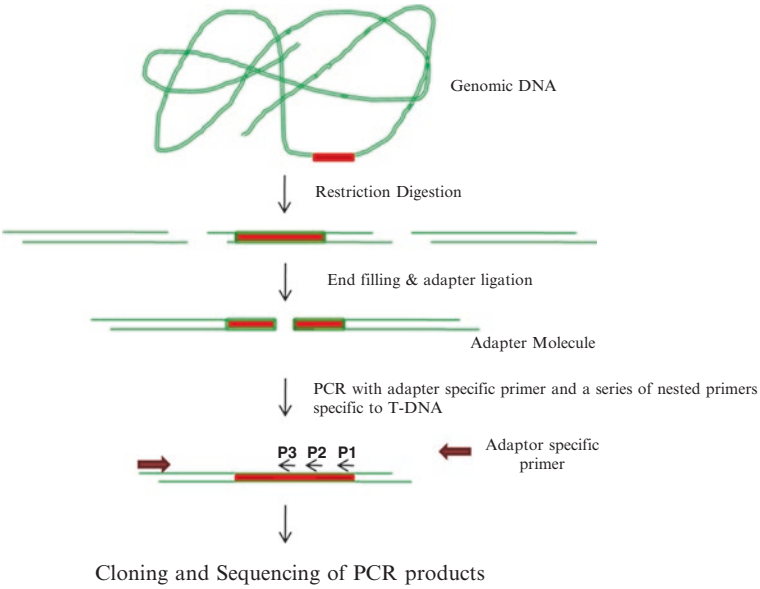
Genomic DNA
Restriction Digestion
End filling & adapter ligation
Adapter Molecule
PCR with adapter specific primer and a series of nested primers specific to T-DNA
P3 P2 P1
Adaptor specific primer
Cloning and Sequencing of PCR products

Fig. 5.5 Schematic representation of adapter ligation-mediated PCR for insertion mutant analysis

adapter and T-DNA; and sequencing of the T-DNA/genomic junction to enable identification of the insertion site in the genome. In most cases, the sequenced genomic region extends to the T-DNA border, enabling the exact location of the insert to be identified (Fig. 5.5). This method has been developed by O'Malley et al. (2007) and used to screen a mutant library and identify over 150,000 T-DNA insertional mutants.

5.4.3 Inverse PCR

Inverse PCR (IPCR) was first described by Ochman et al. (1998). It is used when only one internal sequence of the target DNA is known and flanking DNA sequences of genomic inserts are to be identified. The genomic DNA of the mutant is cleaved with appropriate restriction enzyme (that has a unique site in the T-DNA construct), and the cleaved products are circularized (intramolecular circularization) by ligation. These circularized products form the template for PCR. It is akin to standard PCR but with the primers oriented in the reverse direction as compared to normal PCR (Fig. 5.6). PCR products are sequenced to identify the flanking sites. In promoter trapping, a set of nested primers that are designed from the border sequences of the T-DNA are used for amplifying the flanking DNA.

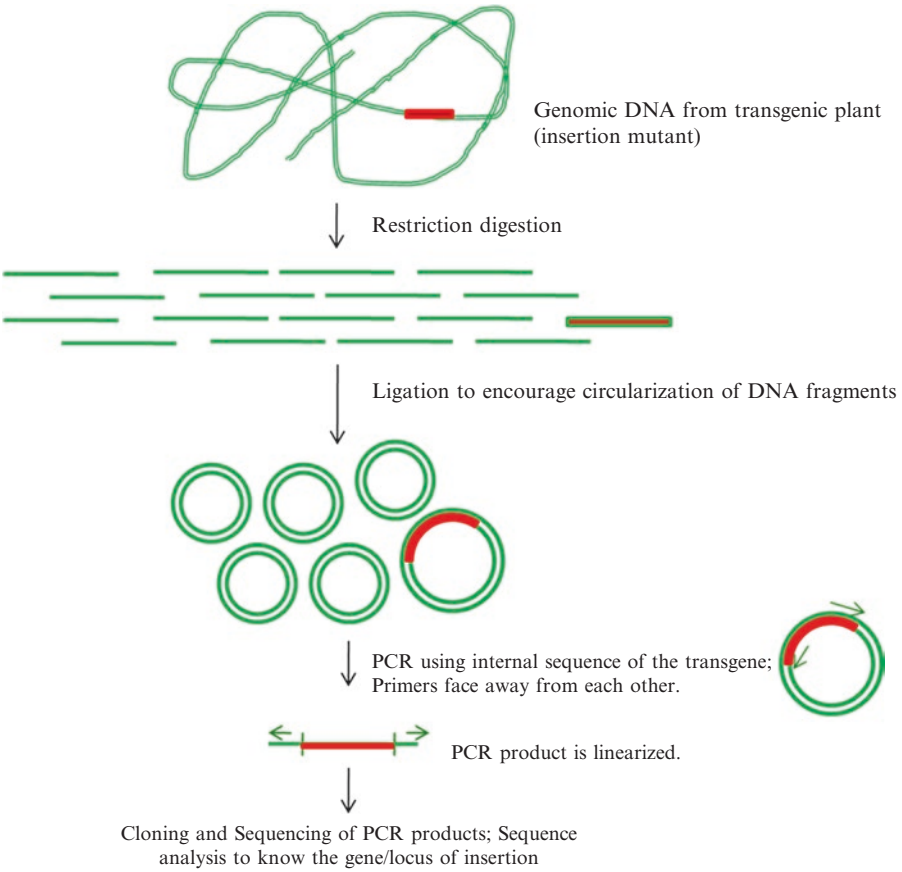
Genomic DNA from transgenic plant
(insertion mutant)
Restriction digestion
Ligation to encourage circularization of DNA fragments
PCR using internal sequence of the transgene;
Primers face away from each other.
PCR product is linearized.
Cloning and Sequencing of PCR products; Sequence
analysis to know the gene/locus of insertion

Fig. 5.6 Schematic representation of inverse PCR for insertion mutant analysis

5.4.4 TAIL-PCR

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) is the most efficient, sensitive and powerful method among all the tools for the recovery of DNA fragments adjacent to known insertion sites/sequences. The method was originally developed by Liu and Whittier (1995), to expedite amplification and sequencing of insert end segments from P1 and YAC clones for chromosome walking. It was later modified by many researchers to isolate different insert sequences, for instance, the isolation of 5′ and 3′ flanking sequences of *Pal* and *Pgi* genes from Yams (*Dioscorea*) (Terauchi and Kahl 2000) and the characterization of a seed-specific 2S albumin gene and its promoter (Li and Gray 2005).

In principle, the TAIL-PCR utilizes three nested primers in consecutive reactions together with arbitrary degenerate primers (AD primers), which are designed to differ in their annealing temperatures, so that the relative amplification frequencies of

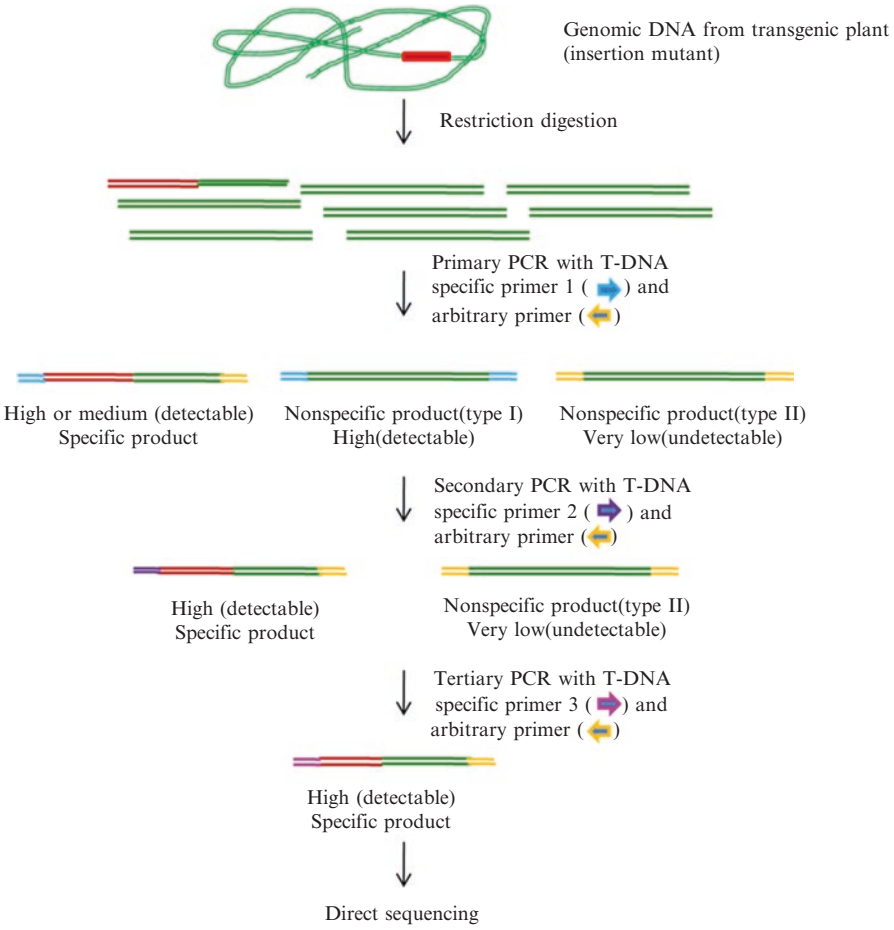

Fig. 5.7 Schematic representation of TAIL-PCR for insertion mutant analysis

specific and non-specific products can be thermally controlled (Fig. 5.7). Alternating cycles of high and low annealing temperature yield specific products bordered by an insertion-specific primer on one side and an AD primer on the other. Further specificity is obtained through subsequent rounds of TAIL-PCR, using nested insertion-specific primers.

In the primary TAIL-PCR of the original method, one low-stringency PCR cycle is conducted to create one or more annealing sites for the AD primer along the target sequence. Target product(s) are then preferentially amplified over non target ones that are primed by the AD primer alone by swapping two high-stringency PCR cycles with one that has a reduced stringency (TAIL cycling). This is based on the principle that in the high-stringency PCR cycles with high annealing temperatures (65–68 °C), only the specific primer with the higher melting temperature can efficiently anneal to target molecules. The AD primer is much less efficient at annealing

due to its lower melting temperature. AD primers with higher degrees of degeneracy, or pooled AD primers, may have more chances to bind to the target sequences. However, this tends to produce undesired smaller products. A simplified version of TAIL-PCR called single oligonucleotide nested (SON)-PCR, which involves only two rounds of PCR with two or three nested sequence primers, can also be used for amplifying flanking sequences (Antal et al. 2004).

The increasing availability of whole-genome sequences renders TAIL-PCR an attractive tool to easily identify insertion sites in large genome tagging populations through the direct sequencing of TAIL-PCR products (Singer and Burke 2003). For large-scale functional genomic approaches, it is desirable to obtain flanking sequences for each individual in the population in a fast and cost-effective manner.

5.4.5 Genome Walking

Genome walking is a simple, PCR-based method to isolate flanking genomic segments adjacent to a known sequence such as cDNA or a known insert such as T-DNA. This method has been used to isolate promoters in plants like sugarcane (Damaj et al. 2010) and *Vigna radiata* (Yang et al. 2011). In this method, uncloned genomic DNA is digested with various restriction endonucleases and ligated to long suppression adapters. The desired genomic region is amplified with a primer specific to the outer part of the suppression adapter and a gene-specific primer. Since the adapters are long and the adapter-specific primer is short (the sufficient ratio is 40 to 20 base pairs), the amplification of the whole pool from that single adapter-specific primer is effectively suppressed, and only the fragments of interest are generated during PCR. Two adaptor-based primers are used in subsequent PCR cycles in order to increase the specificity of PCR along with nested sequence-specific primers. In genome walker PCR, both downstream and upstream flanking sequences of the known sequence can be amplified, the direction depending exclusively upon the strand specificity of the gene-specific primer (Fig. 5.8).

In all the above methods, once the flanking sequences are amplified, the PCR products can be eluted from the gel, cloned into appropriate vectors, sequenced and subjected to bioinformatic analysis using the various tools listed in Table 5.2. These sequences can also be used for further functional characterization or validation by cloning them in appropriate vectors. For promoter characterization, invariably, a promoterless reporter gene fused to the putative sequence isolated is used in the construct. The constructs could then be transformed and subjected to either deletion analysis or site-directed mutagenesis or linker-scanning analysis. DNase I footprinting or gel shift assays can also be performed to identify the cis-regulatory sequence where exactly the transcription factor binds. For identification/mapping of exact TSS, techniques such as 5′ rapid amplification of cDNA ends (RACE), S1 nuclease analysis and RNase protection assay are made use of.

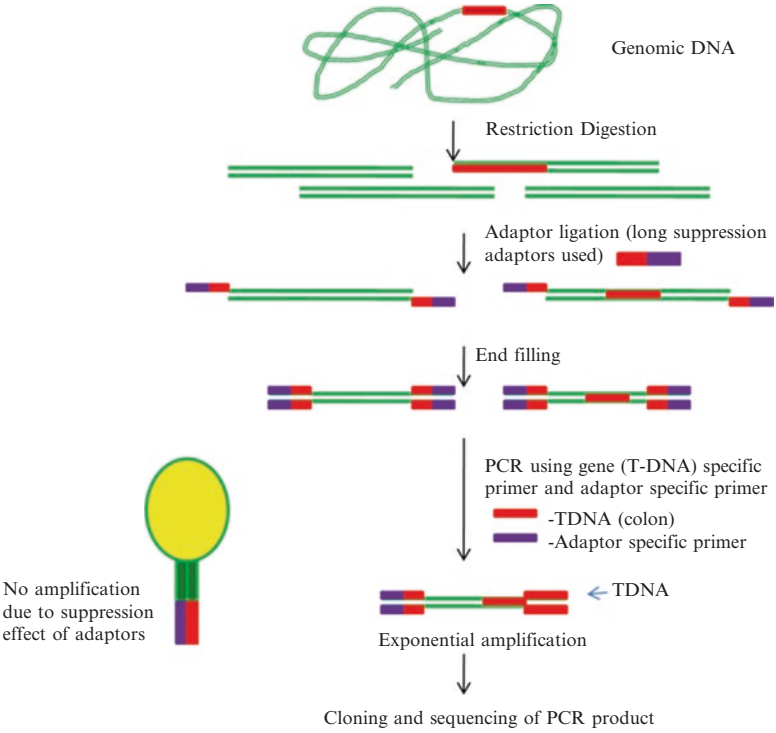
Genomic DNA
Restriction Digestion
Adaptor ligation (long suppression adaptors used)
End filling
PCR using gene (T-DNA) specific primer and adaptor specific primer
-TDNA (colon)
-Adaptor specific primer
No amplification due to suppression effect of adaptors
TDNA
Exponential amplification
Cloning and sequencing of PCR product

Fig. 5.8 Schematic representation of genome walking PCR technique for insertion mutant analysis

5.4.6 Deletion Analysis

This is the most widely used analysis for promoter characterization across prokaryotes and eukaryotes. In this approach, a series of promoter deletion fragments are created and fused to a reporter gene such as uidA or luciferase and transformed. The reporter activity is assayed in the transgenic plants to identify the functional regulatory region. Earlier, the plasmids which harbour the promoter region to be analysed were linearized and digested with either restriction enzyme alone or coupled with exonuclease III digestion (which can digest DNA from 5′ end). This was followed by S1 nuclease treatment (which can cleave single-stranded DNA) to create deletion fragments with blunt ends so that they can be attached to suitable linkers for cloning. Aliquots at various time points were drawn from the digested DNA, checked for size and then cloned into vectors for promoter analysis. However, now, PCR-based methods, where well-defined portions of promoter regions can be easily amplified using appropriate primers, are in vogue for creating deletion fragments (Kato and Hashimoto 2007; Zheng et al. 2004).

5.4.7 Site-Directed Mutagenesis

This is a fundamental tool of modern biology which helps to carry out the functional assay of structural basis of gene and regulatory regions in the genome by base substitution. Several techniques have been described in literature for creation of site-specific mutations. However most of them suffer from the problem of lengthy procedures and a very low frequency of mutants compared to the wild type. Introduction of overlap extension PCR for mutagenesis has overcome these problems. It involves two rounds of PCR to introduce desired mutations in the promoter construct. Two sets of primers are designed—each set having one external and one internal primer. The internal primers are overlapping and complementary in their 5′ end. They have the desired base substitution. The first round of PCR reactions with the two primer sets is carried out separately, and the products are mixed, denatured and reannealed after which the second round of PCR is carried out with the external primers. The PCR amplicons from second PCR are used for cloning into vectors for further analysis. The disadvantage of this method is that base substitutions can be designed only to those regions which are already known to be important in regulation; otherwise, theoretically speaking, as many constructs as the length of the promoter are required to analyse promoter activity.

5.4.8 Linker Scanning

The basic strategy of linker-scanning approach is to create a cluster of point mutations (~10 bp) throughout the promoter DNA cloned into a plasmid vector. This is a more systematic approach with no presumption about the location of functional motif in the promoter. In the original procedure (McKnight and Kingsbury 1982), a series of deletion fragments from both left and right direction using partial exonucleolytic digestion were generated which were linked to a replacement fragment of 10 bp containing a restriction site. The appropriate right and left fragments with the linker DNA are joined to regenerate the original sequence at every position except for the replacement fragment placed anywhere in the entire sequence. Alternatively, synthetic oligonucleotides can also be used to fill in the deleted sequences.

Later PCR-based linker-scanning methods were developed which were faster and easier than the original method. Three general methods, viz. overlapping PCR, asymmetric PCR and circular PCR methods which differ in the number of PCR cycles and oligonucleotides required, have been described in the literature. Among them overlapping PCR is the most common method involving three PCR reactions and four oligonucleotides to create point, insertion, deletion and substitution mutations at any point along the length of the promoter sequence. The first PCR reaction uses a fixed 3′ end primer, but a set of 5′ end primers contain mutation in the middle of the primer. The second PCR uses only the 3′ end primer annealed to the ladder of amplified products obtained in the first PCR amplifying single-stranded products.

These single-stranded products are used as the 3′ end primer which is allowed to pair with a single 5′ end primer in the third PCR to regenerate full-length promoter sequences with mutated clusters.

5.4.9 Footprinting Technique

Unlike the three techniques described so far, footprinting technique does not require transgenic development and assay of the mutated fragments of the promoter construct. It is a gel-based experimental technique wherein either DNase I or dimethyl sulphate (DMS) is used to identify the TF-binding site. In this technique, the promoter sequence is labelled, incubated with total nuclear extract (that contain the TFs) and subjected to DNase I digestion. This sample is subjected to electrophoresis on a denaturing polyacrylamide (PAGE) gel alongside labelled untreated promoter sequence subjected to DNase I cleavage. Since DNase I nucleolytic cleavage is not possible in the region where TF is bound to the promoter, the resultant banding pattern in this region between the treated and untreated samples would be different. Thus, based on the comparative banding pattern, the TF-binding region in the promoter sequence can be identified. The only disadvantage of this technique is that the TF binding is done *in vitro*, which does not reflect the true scenario, since the particular TF may be tissue/stimuli/stage specific and thus not present in the nuclear extract used for analysis.

Instead of DNase I, the promoter fragment incubated with total nuclear extract can also be treated with DMS to methylate the ‘G’ nucleotides present in the sequence followed by cleavage at the methylated bases with piperidine. The rest of the procedure and principle is the same as in the other footprinting assay, i.e. the cleaved samples are run in denaturing PAGE gel alongside the untreated samples. Since the region where TFs are bound cannot be methylated by DMS, the differences in the resulting banding pattern reveal the site of TF binding. The advantage of DMS-based technique is that it can be carried out *in vivo* as well. It can also identify complex interactions wherein multiple TFs bind to different regions of a single promoter (Li and Hall 1999). That’s why this technique is also known as ‘genomic footprinting’ (Busk and Pages 2002).

For genomic or *in vivo* footprinting along with either technique, ligation-mediated PCR (LM-PCR) can also be used. LM-PCR is a useful technique when only one end of the sequence to be amplified is known and has been extensively used in gene isolation, genomic sequencing, chromatin structure and *in vivo* methylation analysis. Mueller and Barbara (1989) first used it for *in vivo* footprinting of muscle-specific enhancer. After DNase I treatment or DMS treatment followed by cleavage, three rounds of PCR reactions are carried out. The unique feature of LM-PCR is that at one end, an oligonucleotide linker is attached and a linker-specific primer is used for that end, and three promoter-specific primers are used from the other end. The first PCR reaction uses only the gene-specific primer on the DNase I or piperidine-cleaved templates generating molecules that have blunt ends

on one side. Linkers are ligated to the blunt ends of the first PCR products and subjected to second round of exponential PCR using a linker-specific primer and a gene-specific primer which is internal to the primer used in the first PCR. In third PCR reaction, end-labelled gene-specific primer and linker-specific primers are used for amplifications. The amplicons are resolved on a sequencing gel to identify the TF-binding regions (Pfeifer and Rigg 1993).

5.5 Plant Promoters in Transgenic Plant Development

Transgenic development can be for crop improvement *per se* or for molecular farming which is a safe, convenient and inexpensive means for the mass production of therapeutic molecules such as vaccines and recombinant molecules for treatment to chronic and life-threatening diseases. This section first describes the use of promoters in molecular farming and then the development of agricultural and horticultural transgenic crops.

Pharmaceuticals and industrial proteins are the main compounds produced using molecular farming. Other metabolic compounds are also produced with the help of this technology, but comparatively on a limited scale. Several recombinant proteins such as industrial enzymes, vaccines, therapeutic proteins and nutraceutical compounds have been successfully produced in plants (Ma et al. 2003; Lienard et al. 2007; Sun 2008). The technology benefits from having low risk of human and animal pathogen contaminations, excellent stability and a relatively high protein yield (Lau and Sun 2009). In addition, it has fewer regulatory hurdles, compared with those of genetically modified crops, and is therefore easy to reach the market in quite a little time period. Avidin, trypsin, beta-glucosidase, peroxidase and lactase were few of the first lot of products that reached the market through this technology.

The plant molecular farming has, thus, opened up an area where plants can be used as bioreactors for production of desired products. Until now, cereals including rice, barley, maize and wheat have been the leading plants where seeds are used as platforms for seed-specific production of recombinant proteins. Among different plant-based systems, seed is the mostly used platform for molecular farming, since it is often considered as a stable source of target proteins. Other plant parts, like leaves and stem, are used but with lesser frequency.

For successful and efficient production of recombinant protein, it is necessary to select a proper promoter, a key step in the optimization of the production process of plant machinery. Since promoters drive the expression of transgene in a particular tissue or at a specific stage, they form basis of molecular farming. As mentioned earlier, the seeds have been the prime target for synthesis of recombinant protein, and, therefore, seed-specific promoters have emerged as a superior choice. This led to the identification, cloning and testing of many seed-specific promoters for recombinant protein expression. The main endosperm-specific promoters in cereals include those of rice globulin, barley D-hordein and maize zein. Those of dicots include *Brassica* napin, soybean lectin, soybean glycinin, β-conglycinin, α' subunit and pea legumin. Performance of seed-specific promoters would differ depending

on the system used, and therefore it would be better to use monocot- or dicot-specific promoters. Maize polyubiquitin-1 promoter is the frequently used promoter in monocot, whereas CAMV 35-S is the most used promoter in dicots. However, these are constitutive and would cause poor growth of the plants owing to constitutive expression of recombinant proteins.

Besides the choice and efficiency of a promoter, protein stability and accumulation after its translation in plant cells are very important in molecular farming. Targeting protein to a subcellular location like endoplasmic reticulum or vacuoles is known to have significant impact on yield (Ma et al. 2003). In addition, fusing a recombinant protein to a plant protein, through genomic modifications, is known to stabilize the recombinant protein in a cell, thereby increasing its production by severe folds.

Enormous benefits in terms of socioeconomic nature as well as environment are expected from transgenic technology in agriculture, i.e. crop improvement *per se*. There are also concerns raised by many researchers regarding its biosafety to environment as well as the consumers, which are legitimate (Nelson 2001). Many of these concerns could be resolved by appropriately engineering the plants to express the transgene in right doses at right time and in right tissues. The promoters that drive and fine-tune the transgene expression could be a major part of the answers to expression-related biosafety concerns (Potenza et al. 2004).

Though single gene-based transgenics are more common now, in the future multigene-based transgenics could become a rule rather than exception, especially, wherever metabolite pathway engineering or tackling complex traits such as tolerance to different abiotic stress and durable resistance to pathogens and insects are concerned (Hunt and Maiti 2001). In such complexly designed constructs, identical promoters would induce transgene silencing (Smirnova et al. 2012). Here again the role of promoters would be very important to enable coordinated and targeted expression of the multiple transgenes. Multicellular organisms have alternate promoters for gene regulation, and search for such alternate promoters is negligent in plan, though search for alternate first exons has picked up. The set of promoters commonly used in vectors and gene constructs are very limited even today (Smirnova et al. 2012). However searching for such promoters from literature and promoter databases is a tedious task, since the information available is too large to sift through. TransGene Promoter Database (http://wwwmgs.bionet.nsc.ru/mgs/dbases/tgp/home.html) developed by Smirnova et al. (2012) describing *cis* elements that provide specific expression patterns and demonstrated in transgenics through reporter gene expression will be very useful in this regard.

The following are the successful examples of transgenics developed for crop improvement which have used promoters other than constitutive ones.

5.5.1 Genetically Engineered Male Sterility in Mustard

Brassica juncea is the second most important oilseed crop of India for which the national average productivity is hovering around 1.0 t/ha in the past decade. Though it is an often self-pollinated crop and hybrid vigour is much known in the crop,

hybrids have not been commercialized successfully. One of the major reasons for this is that the handmade hybrid seeds are weak and fail to show hybrid vigour. Though cytoplasmic male sterility (CMS) systems are well known in this crop and its wild relatives, owing to lack of efficient restorers, hybrid development has not yet used commercially in this crop. Genetically engineered male sterility using barnase–barstar system developed by Mariani et al. (1992) in tobacco and rapeseed is an alternative for cytoplasmic–genetic male sterility (CGMS) which can be engineered in any background for hybrid seed production. Robert Goldberg and his team from Aventis commercialized this in 1996 for canola (*Brassica napus*), an oilseed crop grown widely in the western world. Since then, high-yielding transgenic hybrid canola cultivation has expanded to over a couple of million hectares in North America and Australia. Since herbicide tolerance is an inbuilt component of this system to select for desirable male-sterile plants, appropriate combinations of hybrids and herbicides are available in the market. Roundup Ready is a transgenic canola resistant to M/s Monsanto's herbicide Roundup, and M/s AgroEvo's transgenic canola is resistant to their herbicide Liberty. Later maize has also been engineered with barnase–barstar male sterility for hybrid seed production.

To confer male sterility, the plants are transformed with barnase gene isolated from *Bacillus amyloliquefaciens*, encoding for a ribonuclease called barnase. Barnase is driven by TA29 promoter which expresses specifically in the tapetal cells that nurture anthers, thus preventing pollen production. The transgenics thus developed are male-sterile (MS) lines. The barstar gene, also isolated from *Bacillus amyloliquefaciens* that encodes a barnase inhibitor, which binds to ribonuclease and inactivates it, is engineered in a different background and the lines thus developed are restorer of fertility (RF) lines. Expression of the barstar gene is also restricted to the anthers. For efficient restoration of male fertility in Indian mustard, chimeric promoters combining two anther specific promoters, namely, TA29 and A9 have been evaluated and found to be more efficient than with barstar under the control of TA29 alone (Bisht et al. 2004). Crosses of the MS line with the RF line ensure the production of fully fertile hybrids (F_1 seeds) for commercialization as the expression of the barstar inhibits barnase from destroying pollen (Fig. 5.9).

For commercial seed production, it is important that there is a continuous supply of male-sterile barnase lines. To enable this, the transformed barnase–bar in hemizygous condition is crossed with its untransformed counterpart which gives rise to both type of plants (male sterile and male fertile) upon segregation. The herbicide tolerance trait (bar gene driven by CAMV 35S, a constitutive promoter conferring resistance to phosphinothricin herbicide) is used as a selection tool here, to eliminate unwanted male fertile genotypes in the field by spraying an appropriate herbicide. Thus separate male-sterile and fertility restorer lines are developed through genetic engineering in appropriate genetic backgrounds (which exhibit hybrid vigour upon crossing) for commercial seed hybrid production to emulate the natural phenomenon of heterosis.

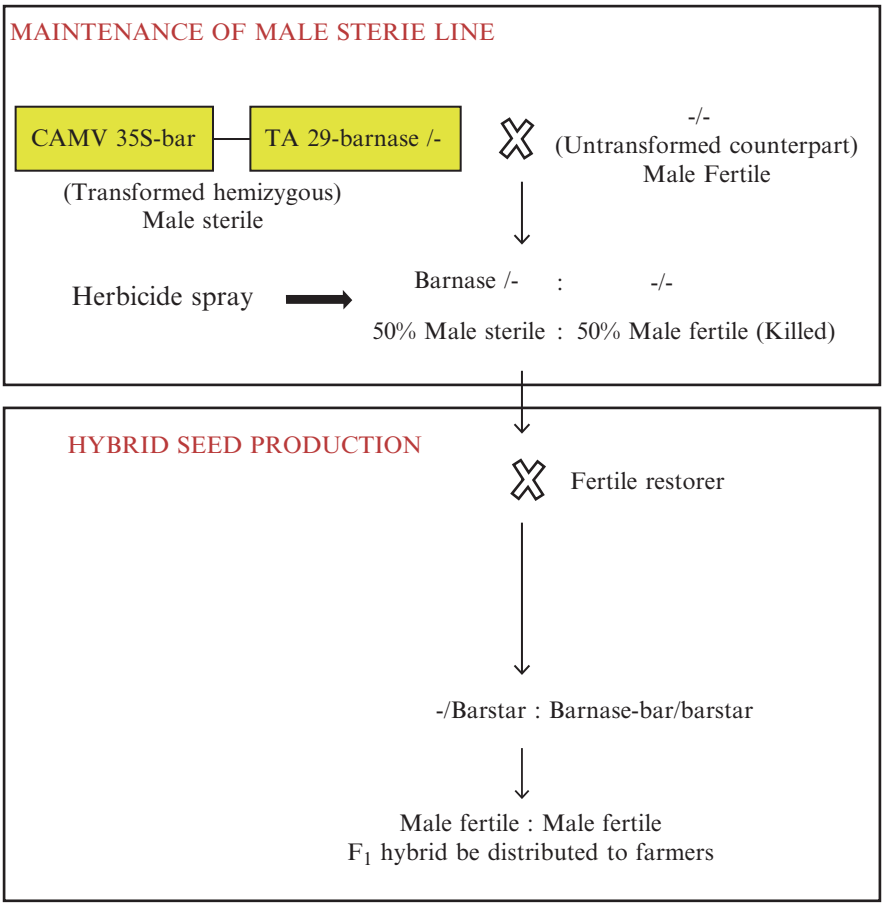

Fig. 5.9 Schematic representation of the use of barnase–barstar system for commercial hybrid seed production

5.5.2 *Transgenic Canola with Enhanced Drought Tolerance*

Abscisic acid (ABA) is one of the phytohormones involved in abiotic stress such as drought, salinity and heat-related signalling in plants apart from germination and seed dormancy. Plants have evolved different adaptation mechanisms to survive moisture deficit, and restricting transpiration loss is one among them. Under moisture stress, the endogenous level of ABA is known to increase resulting in stomatal closure through a complex signalling cascade thus restricting water loss (Blatt 2000). ERA1 (enhanced response to ABA) gene of *Arabidopsis* encodes the β-subunit of farnesyl transferase (FTA), a negative regulator of ABA action (Beaudoin et al. 2000). The *era1* mutant and thus the antisense ERA1 transgenics have enhanced sensitivity to ABA in stomatal guard cells and, hence, reduce

transpirational water loss during drought (Pei et al. 1998). Since in these era1 mutants, the guard cells remain closed all the time, plants remain stunted and are highly compromised for growth and seed yield. However, when this gene is under the control of Rd29, a drought-inducible promoter, and introduced in canola, the stomatal closure occurs only when the plant experiences drought. Transgenic canola thus developed had normal growth and performed well under moisture stress with consistently higher yield (Wang et al. 2005). Multilocation trials also confirmed 15–25 % higher yield than the non-transgenic controls. This technology is being commercialized under the name of Yield Protection Technology (YPM™) in many crops such as maize, cotton, soybean and some ornamentals.

Promoter of *Arabidopsis* hydroxyl pyruvate reductase (*AtHPR1*) expresses specifically in the shoot and not in non-photosynthetic tissues such as root and, hence, used for driving the antisense expression of ERA1 (Wang et al. 2009). The promoter region of *AtHPR1* contains the core motif of the well-characterized dehydration-responsive *cis*-acting element in addition to being shoot specific. Thus conditional and specific downregulation of FTA in canola using the *AtHPR1* promoter driving an RNAi construct of ERA1 exhibited yield protection against drought stress in the field (Wang et al. 2009).

5.5.3 High-Laurate Canola

A recombinant canola variety high in laurate, typically found in tropical oils, was developed by Calgene (now Monsanto) and commercialized in the USA and Canada in 1994 and 1996, respectively. The high-laurate canola is trademarked ‘Laurical’ and sold to the confectionery market as an alternative to cocoa butter. Lauroyl-ACP thioesterase (TE) gene from California bay laurel (*Umbellularia californica*, which is an alternative source of the spice ‘bay leaf’) was engineered to express in canola, leading to modified seed fatty acid content, specifically high levels of lauric acid. For identification of gene sequence responsible for encoding TE in *U. californica*, the enzyme was extracted from source tissue, purified and sequenced. From the amino acid sequence of the enzyme, the nucleotide sequence was identified in reverse, and a unique DNA probe was constructed. The genomic DNA and cDNA of *U. californica* were hybridized with the unique probe to identify the gene. Once the gene expression was confirmed in bacteria and model plant, it was used to transform canola using *Agrobacterium*-mediated transformation (Del Vecchio 1996). The introduced thioesterase enzyme was placed under the control of napin promoter and is active in the fatty acid biosynthetic pathway of the developing seed causing accumulation of triacylglycerides containing esterified lauric acid and, to a lesser extent, myristic acid. The increased levels of lauric acid in oil from the modified canola lines allow for its use as a replacement for other lauric acid oils, such as coconut and palm kernel oil, in products such as confectionery coatings and fillings, margarines, spreads, shortenings and commercial frying oils.

5.5.4 Golden Rice

Golden rice is a fine example of genetic engineering in introducing a functional pathway which only partially existed in nature, that too driving the expression in a specific target tissue, though the product is not commercialized as yet. Rice endosperm does not have carotene, and engineering rice for β-carotene can alleviate vitamin A deficiency prevalent in Southeast Asia and Africa. By engineering two genes, namely, phytoene synthase (*psy*) from daffodil and carotene desaturase (Crt1) from *Erwinia uredovora* (now known as *Pantoea ananatis*) into a *japonica* rice cultivar, it was demonstrated that rice endosperm can be made to accumulate β-carotene (Ye et al. 2000). Both these genes were placed under the control of glutelin promoter, a seed-specific one so as to direct the expression only in grains. The downstream genes in the pathway, lycopene isomerase and lycopene cyclase, were found intact in rice thus making golden rice engineering easier. Later these genes were also introduced in *indica* rice cultivar successfully (Hoa et al. 2003). Golden rice 2 which could accumulate 31 μg/g β-carotene as against the 1.6 μg/g β-carotene produced earlier was developed by employing maize PSY genes under the control of the same rice glutelin promoter (Paine et al. 2005).

5.5.5 Drought-Tolerant Maize

Maize transformed with *Bacillus subtilis* cold-shock protein B (cspB) driven by the rice actin promoter gave significant yield advantage over their untransformed counterparts (Castiglioni et al. 2008). Though no yield was observed under normal water supply, yield advantage was observed only under severe water stress which normally reduces yield by more than 50 %. However the transgenic hybrids are being commercially developed intended for suboptimal maize farming systems (Yang et al. 2010). It was intended for commercial release under the trade name of DroughtGard in 2013.

Freeze-tolerant eucalyptus has been developed by transforming it with C-repeat-binding factor 2 (CBF) gene from *Arabidopsis* driven by cold-inducible rd29A promoter (Zhang et al. 2012).

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Chapter 6
Metabolic Engineering of Secondary Plant Metabolism

Usha Kiran, Athar Ali, Kamaluddin, and Malik Zainul Abdin

Abstract Metabolic engineering is a modulation of metabolic pathway(s) of the host either to increase the concentration of existing compounds or to produce a novel compound. It began with the engineering of microorganisms, and the concept has been extrapolated to plants opening newer promising perspectives. The high-value secondary metabolites such as drugs (e.g. paclitaxel, artemisinin and vincristine), dye and pigments, flavour and fragrances and food additives are the main target. Cloning and expression of gene(s) in host plant allow partial/complete reconstitution of biosynthetic pathways.

A major challenge for the commercialization of high-value secondary metabolites is their low production levels *in planta*. The continual demand for novel molecules with new or superior biological activities by the industry in recent years has resulted in a great interest in secondary metabolism. Metabolic engineering and pathway optimization with the aim to reduce costs and increase productivity are, therefore, the main focus of academia and industry.

Redirecting or stimulating a reaction/pathway requires an insight into the endogenous pathway(s) to understand the best intervention point(s). This chapter thus discusses the strategies developed to overcome bottlenecks for enhancing the production of high-value products in a heterologous background, without harming the host plant.

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6.1 Introduction

Plant metabolic engineering involves the modification of endogenous pathways to increase flux towards particular desirable molecules. It is generally defined as the redirection of one or more enzymatic reactions so as to produce new compounds, improve the production of endogenous high-value compound(s) or mediate the degradation of endogenous unwanted compound(s). The development in genomics and proteomics to elucidate and characterize metabolic pathways in the past decades has made metabolic engineering a versatile tool for manipulation in host genome. Further, modelling of metabolic pathways showed that these pathways are controlled at multiple levels and any form of alteration can have wide-ranging effects on the host at metabolic as well as genomic levels (Zhu et al. 2008).

Secondary metabolites are complex biomolecules which is non-essential for housekeeping functions; however, their presence provides adaptive advantages to plants by modulating the behaviour of microbes, pests and animals. Due to inherent unique properties of these secondary metabolites such as attracting pollinators, repelling pests and pathogens, the host plants have been used by humans. These pharmacologically active molecules can be used as flavours, fragrances and crop protection products (Miralpeix et al. 2013). These secondary metabolites from plant origin can be classified on the bases of their biosynthesis as alkaloids, terpenoids and phenolic compounds. Five-carbon precursor isopentenyl diphosphate (IPP) is the precursor for all terpenoids. The carbon backbone for alkaloids, which contain one or more nitrogen/sulphur atoms, is derived principally from aromatic amino acids. The phenolic compounds are formed either by shikimic acid pathway or the malonate/acetate pathway.

Over the past few decades, whole plant and plant cell culture has been extensively used for natural or recombinant compound productions for commercial utilization. Further, the association of industry and agriculture became possible due to the use of metabolic engineering for the modulation of secondary metabolite concentration which can be used as biosynthetic precursors, biodegradable polymers of plant origin and medicinally active compounds (Verpoorte and Alfermann 2000). Extracted from plants, these bioactive compounds are used in pharmaceutical, agrochemical, flavour and fragrance, food additive and pesticide industries. Derivation of plants with novel traits through metabolic engineering is another goal of this association paving the way for newer products in agriculture, environmental applications and production of chemicals.

To manipulate the plants or plant cell cultures for enhanced or novel secondary metabolite production, the development of an integrated information system at genetic, cellular and molecular levels is a prerequisite. With better understanding of these extensive, integrated and complex metabolic pathways, manipulation of enzyme(s) to produce the high-value-desired end product would be easy. The insight into pathways would facilitate the manoeuvring of steps or pathways which leads to production of unwanted metabolites also. This would channelize the carbon flux for the synthesis of desired metabolite and thus enhancing its concentration. For generating such information, research should be focused on elucidating the complete pathways that catalyse secondary metabolite formation, in terms of both enzymes

and intermediates (metabolic pathway profiling). The next step is the selection of targets in terms of genes, enzymes and compartments. These integrated studies would ultimately lead to the novel transgenic plants or plant cell cultures with desired compounds in higher concentration. Apart from direct applications of such plants to academia and agricultural industries, the knowledge gained would give an understanding about the adaptive/functional roles of secondary metabolism in plants.

6.2 Plants as Pharmaceutical Factories

Plants are a rich source of bioactive compounds which are used either as drugs or precursors of semisynthetic drugs. They may also serve as valuable precursors for novel drugs. In the last 3 decades, 1/3 of the ~ 980 new pharmaceuticals originated from or was inspired by natural products and ~ 25 % of the new drugs approved was based on phytomolecules (Hendrawati et al. 2010). Recent reports suggest worldwide, close to 50,000 plants are used for medicinal purposes. According to WHO report, around 80 % of the world's population especially developing world population use herbs as medicines. The use of herbs is viewed as an integral part of healthcare and traditional medical practice in these communities (Bodeker et al. 2005). According to Global Industry Analysis report, with the increasing consumer awareness and acceptance of natural therapies, the global herbal medicines and supplements market is predicted to reach $107 billion by the year 2017.

Whole plant, crude extract and pure compound extracted from plants thus play an important role in contemporary pharmacy and medicine. Typical active plant compounds (Table 6.1 and Fig. 6.1), which are commonly used as drugs, include the terpenoids, flavonoids, polyketides, alkaloids and phenylpropanoids. The pure phtyocompounds such as morphine (*Papaver somniferum* L.), artemisinin (*Artemisia annua* L.), paclitaxel (*Taxus brevifolia* Nutt.), genistein (*Glycine max* L.), scopolamine (*Duboisia* species) and podophyllotoxin (*Podophyllum* species) are used as drugs or precursors of the drugs. Despite the use of and demand for these phtyocompounds, their availability is a major setback for the herbal industries due to the following limitations:

- As these are secondary metabolites, therefore, synthesized by plants in limited amounts.
- Most medicinal plants are collected from the wild which makes collection difficult. Slow growth and intensive collection from the wild are making some plants close to extinction. The current extinction rate of these plants is estimated to be 100–1000 times higher than for non-medicinal plants, and this number is expected to increase in the future.
- They may be restricted to one species or genus and produced only during a particular stage of growth. The synthesis may occur during specific season, stress or nutrient availability conditions.
- Uneconomical synthesis of these plant-derived compounds via organic chemistry due to their chemical complexity and specific stereochemistry.

Table 6.1 Overview of the production of the plant-derived and medicinally relevant compounds

Compound	Activity/function	Plants source	Reason for combinatorial biosynthesis
Dihydroartemisinic acid (terpenoid)	Antimalarial	*Artemisia annua*	Availability in nature is limited.
			Chemical synthesis is economically not feasible
Paclitaxel (terpenoid)	Antitumor	*Taxus* spp.	Low concentration in plant.
			Slow growth.
			Source nonrenewable – harvesting the bark results in the tree death.
			Chemical synthesis uneconomically
Podophyllotoxin (lignan)	Antitumor	*Podophyllum* spp.	Endangered species.
			Chemical synthesis is economically not feasible
Scopolamine (alkaloid)	Anticholinergic	*Duboisia* spp.	Multiple chiral centres.
			Chemical synthesis is economically not feasible
Morphine (alkaloid)	Analgesic	*Papaver somniferum*	Multiple chiral centres.
			Chemical synthesis is economically not feasible
Vincristine (alkaloid)	Antitumor	*Catharanthus roseus*	Multiple chiral centres.
			Chemical synthesis is
			economically not feasible

Consequently, whole plants compared to cells in bioreactors (plant cell culture) or microorganisms provide a simple, cheap and scalable platform for higher production. Transgenic plants can be grown, maintained and harvested using equipments already available. The choice of a host plant for metabolic manipulations depends on the particular metabolite to be produced and indigenous presence of required precursors. To ease the process or extraction, the target metabolite can be sequestered in specialized organs such as leaf hairs, trichomes or glands (Wagner et al. 2004). There is, however, a growing concern in using cultivated plants for phytochemicals, especially pharmaceuticals particularly in areas where these plants are used for human and animal consumption. Thus, species other than crop plants that do not hybridize with major cultivated plants or with their wild relatives are being genetically engineered to produce economically important secondary metabolites. Further, with the advances in the molecular biology techniques for recombination, cloning, expression, of genetic material and knowledge base of the plant biosynthetic pathways, metabolic engineering may be instrumental in designing methodology to overcome the shortage of such compounds from plant sources.

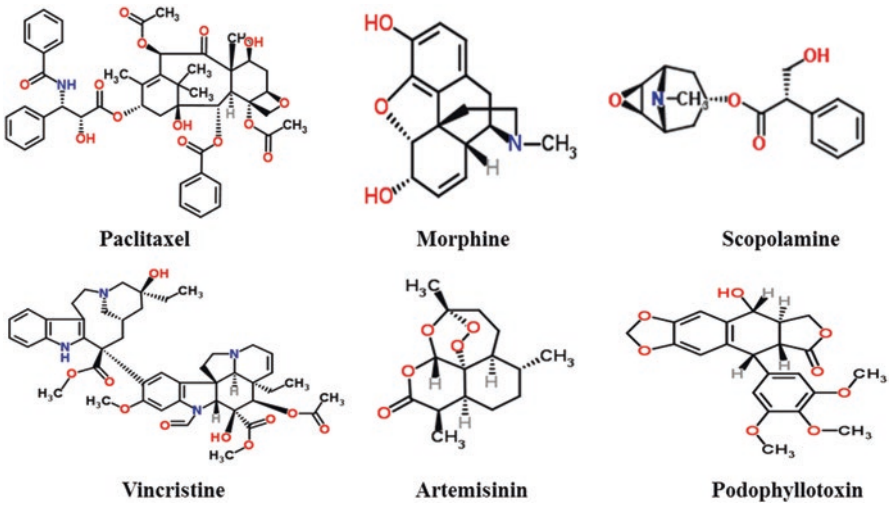

Fig. 6.1 Chemical structures of important plant-derived medicinal compounds

6.3 Strategies for Metabolic Engineering in Plants

The complex organic compounds from primary and secondary metabolism are synthesized through a cascade of enzymatic reactions, and these cascades are often long and convoluted metabolic pathways. Therefore, metabolic engineering has seen a progressive change from single-gene intervention to multigene transformation (Zhu et al. 2008; Halpin 2005). The objectives of metabolic engineering are enhanced production of a specific desired compound, inhibition of the production of a specific unwanted compound and production of a novel compound. These goals could be achieved by upregulation of pathways (overexpression of enzymes), by downregulation of enzymes (introduction of antisense gene and RNAi) and by blocking catabolism, either through increasing the transport of metabolites into the vacuole or downregulation of catabolic enzymes. Thus, modulating multiple enzymes consecutively in a pathway or upregulating enzymes in one pathway while suppressing others in another competing pathway through metabolic engineering could help to control metabolic flux in a more predictable manner (Zhu et al. 2013).

6.3.1 Upregulation of Metabolic Pathway(s) by Overexpression of Enzyme(s)

In a metabolic pathway, there are specific branching points where enzymes compete for a common precursor putting constrains on the flux inflow to a particular pathway. These are followed by committed steps which are regulated by 'rate-limiting or bottleneck enzymes'. Increasing and redirecting the precursor pool towards the

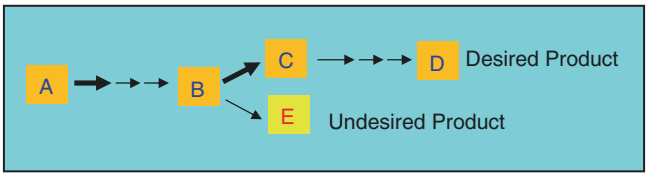

Fig. 6.2 Rerouting the carbon flux to the desired product by overexpression of gene(s) encoding first the branch point enzyme(s). ➞ Overexpressed gene(s)

biosynthesis of the target compounds can theoretically increase the production of the desired secondary metabolites. The characterization of genes encoding rate-limiting enzymes of secondary metabolites and understanding of their spatial and developmental regulation along with their expression have established their important role in developing strategies for genomic and metabolic manipulations. The overexpression of targeted genes can result in increased flux through the pathway, leading to higher production of the secondary metabolites (Muir et al. 2001; Ravanello et al. 2003; Botella-Pavía et al. 2004; Sato et al. 2007) (Fig. 6.2).

The effect of overexpression of gene(s) of enzymes catalysing the various steps in artemisinin biosynthestic pathway to enhance the production of artemisinin in *Artemisia annua* L. plant (annual wormwood or sweet wormwood or sweet annie, Asteraceae) is a well-studied example of this strategy. Artemisinin is a secondary metabolite having a unique sesquiterpene lactone with an endoperoxide bridge and has gained much attention due to its potent antimalarial properties. During research into novel antimalarial drugs in the 1970s, artemisinin was identified as the active ingredient in extracts of the *A. annua* L., Chinese medicinal herb. In recent years, the wide spread of multidrug-resistant malaria has led to promotion of the use of ACTs by the World Health Organization (WHO) as the first line of treatment resulting in growing demand for artemisinin (World Health 2008). The low yield of artemisinin from *A. annua* L. (0.01–1 % of dry weight), however, has led to difficulties in managing its demand for the large-scale production of ACTs (Liu et al. 2006; Abdin et al. 2003). The need of hour, therefore, is reducing and stabilizing the price of this phytocompound by integrating molecular breeding and synthetic biology strategies for its production and yield improvement.

Artemisinin belongs to terpenoid, and knowledge about the general terpenoid and specific artemisinin biosynthetic pathways is fuelling the extensive metabolic engineering of *A. annua* the world over. During the last decade, a number of genes encoding enzymes of artemisinin biosynthesis have been cloned, and a putative biosynthetic pathway has been constructed. Amorpha-4,11-diene (amorphadiene), the aliphatic backbone of this sesquiterpene, is synthesized from farnesyl diphosphate (FDP), which can, in turn, be produced from isopentenyl diphosphate (IPP) (Fig. 6.3). Amorpha-4,11-diene synthase (ADS) is the first committed enzyme of artemisinin biosynthesis pathway (Bouwmeester et al. 1999; Mercke et al. 2000; Wallaart et al. 2001; Alam and Abdin 2011). It converts farnesyl diphosphate to amorpha-4,11-diene and diverts carbon flux from mevalonate pathway into artemis-

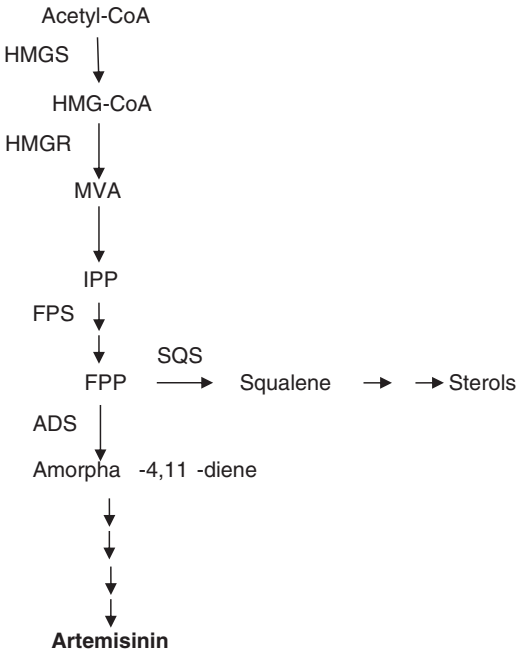

Fig. 6.3 Schematic representation of the mevalonate pathway leading to the biosynthesis of artemisinin, sesquiterpenes and sterols (*ADS* amorpha-4,11-diene synthase, *FPP* farnesyl diphosphate, *FPS* farnesyl diphosphate synthase, *HMG-CoA* 3-hydroxy-3-methylglutaryl CoA, *HMGR* HMG-CoA reductase, *HMGS* HMG-CoA synthase, *IPP* isopentenyl diphosphate, *MVA* mevalonate, *SQS* squalene synthase)

Table 6.2 Enhancement in artemisinin content by overexpression of artemisinin biosynthetic pathway genes in transgenic *A. annua* L.

Genes overexpressed	%/fold enhancement in artemisinin	References
fps	Two to threefold	Chen et al. (2000)
ipt	30–70 %	Sa et al. (2001)
fps	34.4 %	Han et al. (2006)
hmgr	22.5 %	Aquil et al. (2009)
hmgr	38.9 %	Nafis et al. (2010)
hmgr and ads	Sevenfold	Alam and Abdin (2011)

inin production. A number of other genes encoding downstream enzymes, viz., CYP71AV1, DBR2, ALDH1 and RED1, from artemisinin biosynthetic pathway have been cloned and overexpressed in *A. annua* L. by several investigators leading to increased artemisinin content (Chang et al. 2000; Wallaart et al. 2001; Mercke et al. 2000; Aquil et al. 2009; Teoh et al. 2009; Olsson et al. 2009; Zhang et al. 2008; Rydén et al. 2010; Han et al. 2006). The findings of these studies are summarized in Table 6.2. The overexpression of amorpha-4,11-diene synthase (ADS) in addition with the enhanced metabolic flux in FPP building block-synthesizing pathway(s), i.e. the MVA or MEP pathways, by overexpressing hmgr (the rate-limiting enzyme of mevalonate pathway) gene has led to sevenfold enhanced artemisinin content in *A. annua* L. plants (Alam and Abdin 2011), suggesting combinatorial cloning as one of the better options of metabolic engineering to enhance secondary metabolite contents in plants.

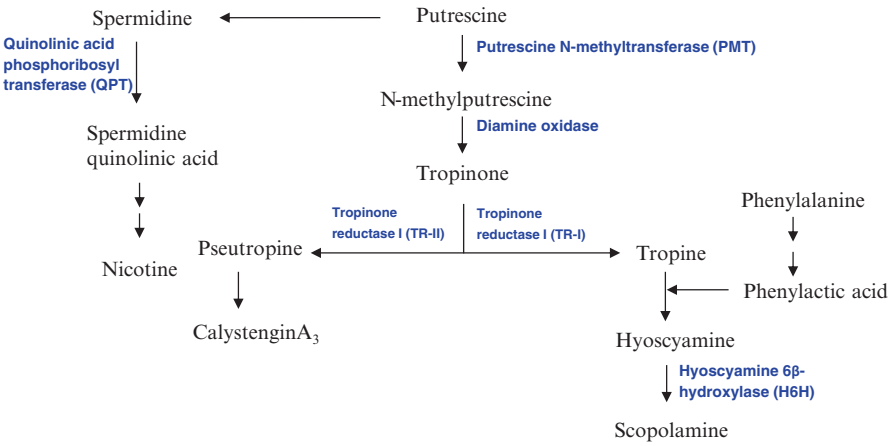

Fig. 6.4 Schematic representation of the tropane pathway leading to the biosynthesis of scopolamine

In another example, the overexpression of key enzyme gene in tropane alkaloid biosynthesis also resulted in enhanced alkaloid contents in transgenic plants. Hyoscyamine as well as its racemate (atropine) and scopolamine (hyoscine) are the more abundant tropane alkaloids in *Duboisia*, *Datura*, *Hyoscyamus*, *Atropa* and *Scopolia* species (Griffin and Lin 2000). However, *Duboisia* leaves are the main source of scopolamine worldwide. The world demand for scopolamine is estimated to be about 10 times greater than hyoscyamine and its racemic form atropine (Palazón et al. 2008) due to its higher physiological activity and fewer side effects.

The putrescine N-methyltransferase (PMT) is the rate-limiting enzyme catalysing the first committed step in the biosynthesis of alkaloids. The formation of N-methylputrescine removes putrescine from the polyamine pool and diverts the methylated compound exclusively towards alkaloid production (Fig. 6.4). The overexpression of PMT in *Scopolia parviflora*, therefore, has led to eightfold increase in scopolamine and 4.2-fold increase in hyoscyamine production (Lee et al. 2005). In another study, Moyano et al. (2002) introduced *pmt* gene of *Nicotiana tabacum* into the genome of a scopolamine-rich *Duboisia* hybrid. The results showed increased levels of *N*-methylputrescine up to fourfold as compared with wild-type hairy roots, without any significant improvement in the tropane alkaloids, hyoscyamine and scopolamine. This study, hence, suggests that though the ectopic expression of tobacco *pmt* increased carbon flux towards the biosynthesis of tropane alkaloids, but due to the limitation of the activities of enzymes catalysing downstream steps in the pathway, the carbon was not fully utilized in the synthesis of these alkaloids (Moyano et al. 2002).

The overexpression of only rate-limiting enzymes in a complex metabolic network may not be sufficient to increase the content of a particular secondary metabolite and may have different biochemical outcomes depending on how, where and when the gene(s) was expressed (Zhang et al. 2004; Liu et al. 2010). The fidelity of

integration of gene may be attributed to the poor understanding of transformation process and transgene integration mechanism into plant genome. The integration of many genes at one or a few loci may happen by chance, and the positional effect of transgene may lead to the difference in the expression levels among the transgenic lines (Matzke and Matzke 1998). The insertion of multiple copies of the transgene may result in post-transcriptional gene silencing either through DNA methylation or co-suppression. The tight regulation of metabolite biosynthesis and accumulation of target products may limit the impact of this approach. Further, the overexpression of single gene may enhance the precursor metabolic pool leading to the enhanced production of undesired products along with the targeted secondary metabolite. Hence, the simultaneous upregulation of more than one biosynthetic gene(s) in the pathway is the preferred strategy.

6.3.2 *Downregulation of Metabolic Pathway(s) by Suppression of the Gene(s) Encoding Key Enzyme(s)*

The bioproduction of a desired secondary metabolite can be enhanced by inhibiting/reducing the flux of the substrate/intermediate towards the biosynthesis of undesired product/competing pathways. This can be achieved by reducing the level of branch point enzyme catalysing the committed step in the production of undesired product using antisense, co-suppression and RNA interference (RNAi) methods (Fig. 6.5).

The silencing of the gene(s) encoding the branch point enzyme(s) can be targeted to specific plant tissues and organs with minimal interference of the normal plant life cycle by using tissue or organ-specific RNAi vectors. Mutants with the RNAi effect have been shown to be stable for at least 20 generations. This methodology was used to enhance artemisinin content in *A. annua* L. plants.

A 30 carbon linear compound formed from two molecules of farnesyl diphosphate (FDP) is the first committed precursor for sterol biosynthesis in plant species including *A. annua* (Goldstein and Brown 1990). This reaction is catalysed by squalene synthase (SQS) serving as a crucial branch point enzyme for regulation of sterol biosynthesis. Thus, by modulating this enzyme control of carbon flux into

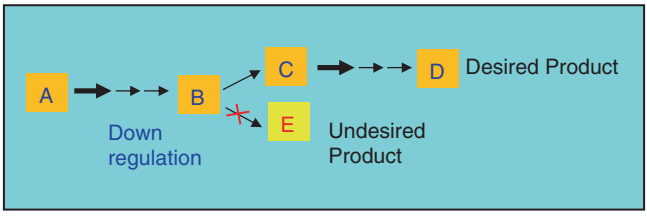

Fig. 6.5 Diverting the carbon flux by blocking the competing pathway by downregulating the gene expression. ➞ Overexpressed gene(s), ✳➞ downregulated gene

non-sterol isoprenoids and sterol, biosynthesis could be achieved. Studies suggest that if the SQS gene expression is inhibited, the carbon flux into sterol biosynthesis may be diverted to sesquiterpenes biosynthesis. Wang et al. (2012) supported the hypothesis and showed that the overexpression of antisense squalene synthase gene in *A. annua* L. plants reduced squalene content and increased artemisinin content in transgenic plants.

In another study, the artemisinin content of *A. annua* L. plants was enhanced by suppressing β-caryophyllene synthase (CPS), a sesquiterpene synthase gene that encodes enzyme catalysing the first committed step in the competing pathway that utilizes the metabolic precursor of artemisinin (Chen et al. 2011). The overexpression of antisense β-caryophyllene synthase gene significantly reduced the expression of endogenous CPS along with the β-caryophyllene content in the transgenic lines as compared to the wild-type plants.

Antisense RNA inhibition approach was also successfully used in *Taxus* × *media* to enhance the production of secondary plant metabolites (Li et al. 2013). Taxol (commonly name paclitaxel), originally derived from the pacific yew (*Taxus brevifolia*), is a complex diterpenoid. It has been approved as an important antitumor and antileukemic drug (Suffness 1993). Taxol interferes with mitotic microtubular dynamics. It arrests dissolution of the mitotic spindle during cell division. Although the uses of taxol are increasing, limited amounts are obtained due to low concentration in the host plant (Exposito et al. 2009). The synthesis of taxol from geranylgeranyl diphosphate (the diterpenoid precursor) involves at least 20 distinct enzymatic steps with a similar number of taxoid intermediates (Hezari and Croteau 1997; Croteau et al. 2006; Ketchum et al. 2003). Both 13α-hydroxylase (13OH) and 14β-hydroxylase (14OH) utilize 5α-hydroxytaxa-4(20),11(12)-diene as a substrate (Ketchum et al. 2007) and, hence, catalyse the branching point reactions of the taxol biosynthesis. The taxoid 14β-hydroxylase (14OH) directs carbon flux of taxol pathway to 14β-hydroxy taxoids (Fig. 6.6).

To increase the production of taxol, Li et al. (2013) used antisense RNA inhibition approach to suppress taxoid 14β- hydroxylase gene (*14OH*) in the *Taxus* × *media* TM3 cell line (Li et al. 2013). An antisense RNA expression vector containing *14OH* from *Taxus chinensis* was introduced into *Taxus* by *Agrobacterium tumefaciens*-mediated transformation. Southern blot analysis of marker gene hygromycin phosphotransferase gene (*HYG*) revealed successfully integration of desired gene into the *Taxus* genome. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed dramatically decrease in levels of *14OH* mRNA in transgenic cells suggesting that endogenous *14OH* gene expression was significantly suppressed by introduced *14OH* antisense gene. Further, the silenced cell lines showed markedly reduced levels of yunnanxane, taxuyunnanine C, sinenxan C, when compared with those of the nontransgenic cell line. Thus, antisense RNA strategy showed to be a useful tool in suppressing the important genes in *Taxus* sp. that divert the precursors/intermediates of taxol pathway to side-route pathways for the synthesis of other/undesired metabolites (Li et al. 2013). Thus, inhibiting the pathway that competes for the precursor of desired secondary metabolite by antisense technology would prove to be an effective means of enhancing its content in natural source.

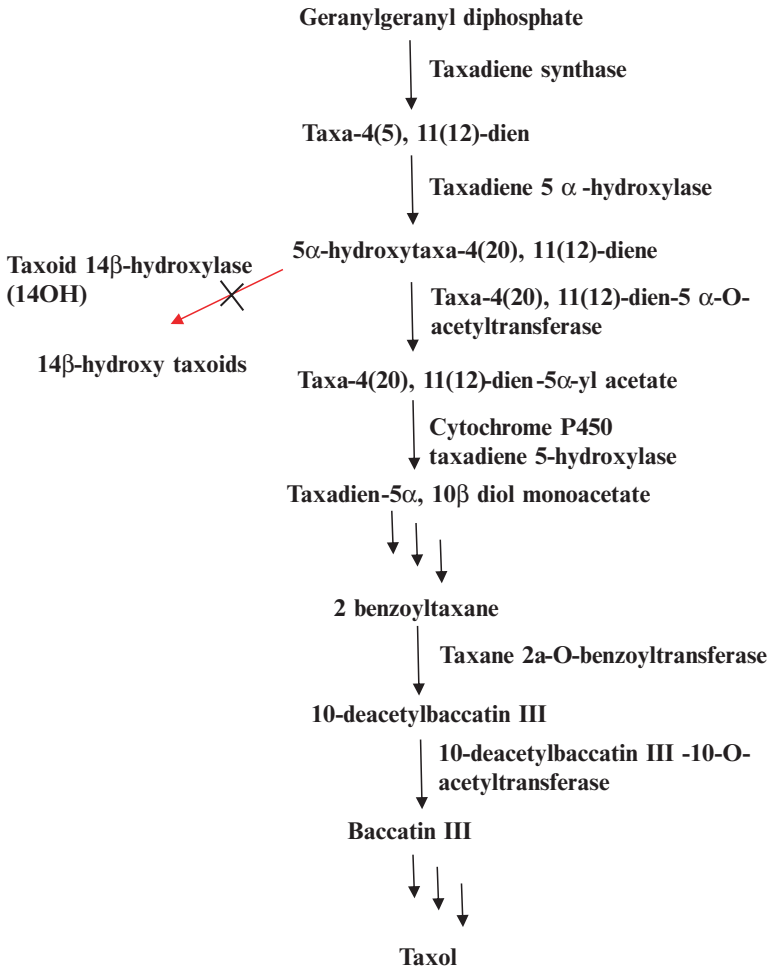

Fig. 6.6 Schematic representation of pathway leading to taxol biosynthesis

6.3.3 Production of a Novel Compound (Synthetic Biology)

Metabolic engineering also provides techniques to transfer a whole metabolic pathway or part of it from native plant to target plant to produce novel compound(s). Thus, synthetic biology describes the de novo assembly of genetic systems using prevalidated components (Haseloff and Ajioka 2009). It utilizes specific promoters, genes and other regulatory elements to create ideal genetic circuits that facilitate the accumulation of a novel metabolite(s). The concept of synthetic biology removes any dependence on naturally occurring sequence(s). Since plants have much more complex metabolic pathways especially for secondary metabolite, most work on synthetic biology has been done with microorganisms (Weber and Fussenegger

2011). In recent years, however, this approach has also been used in plants for growth- and development-related signalling pathways, development of phytodetectors and biofortification of crops (Naqvi et al. 2009; Zurbriggen et al. 2012).

The success of the synthetic biology has been demonstrated by introducing the whole gene cassette in rice for production of carotenoids. Carotenoids are a subfamily of most widely distributed isoprenoids comprising orange, yellow and red natural pigments and synthesized by bacteria, algae and fungi. More recently, carotenoids have received attention for their antioxidant activities and inhibitory role in the inception of chronic diseases (Rao and Rao 2007).

Rice is the main staple food especially in many African and Asian countries. It is generally consumed after removing the outer layers (aleurone, tegmen and pericarp) by milling. The presence of oil-rich aleurone layer turns rancid upon storage and hence has to be removed especially in hot and humid tropical and subtropical areas. The milling leaves only the edible endosperm which is filled with starch granules and protein bodies. Thus, rice grain become devoid of several nutrients essential which are present in outer layer and are essential for the maintenance of health. These include carotenoids exhibiting provitamin A activity. Thus, reliance on rice as a primary staple food contributes to vitamin A deficiency leading to night blindness, a serious public concern in at least 26 countries including Asia, Africa and Latin America with high population density (Sommer 2008). A complementary intervention to existing strategies for minimizing vitamin A deficiencies in these countries is to fortify rice with provitamin A. This aim could only be attained through synthetic biology rather than by conventional breeding, as the rice cultivars lack the key enzymes of provitamin A biosynthetic pathway in the endosperm.

The carotenoid biosynthetic pathway has been completely understood in rice due to extensive genomic studies. With well-established transformation protocols, rice serves as appropriate plant for production of β-carotene through genetic engineering. To engineer the pathway towards β-carotene formation in rice, phytoene synthase (*psy*) and lycopene β-cyclase (β-*lcy*) from *Narcissus pseudonarcissus* (daffodil) with endosperm-specific glutelin promoter and phytoene desaturase gene (*crt*I) from *Erwinia uredovora* (bacteria) with constitutive 35S promoter were mobilized and integrated into the rice genome (Fig. 6.7). The overexpression of these genes resulted in enhanced β-carotene synthesis in rice endosperm (Beyer et al. 2002).

Similarly, *Atropa belladonna* (deadly nightshade), a plant that normally accumulates hyoscyamine precursor of scopolamine, was engineered to produce scopolamine by constitutive expression of H6H (hyoscyamine 6β-hydroxylase) from *Hyoscyamus niger* (henbane). The H6H (EC 1.14.11.11), a 2-oxo-glutarate-dependent dioxygenase, catalyses the hydroxylation of hyoscyamine to 6-hydroxyhyoscyamine followed by 6-hydroxyhyoscyamine epoxidation to scopolamine (Zhang et al. 2004) (Fig. 6.4). The transgenic *Atropa belladonna* was found to accumulate up to 1.2 % scopolamine on dry weight basis. The alkaloid composition of aerial parts of mature plants changed from over 90 % hyoscyamine in wild-type plants to almost exclusively scopolamine in transgenic plants.

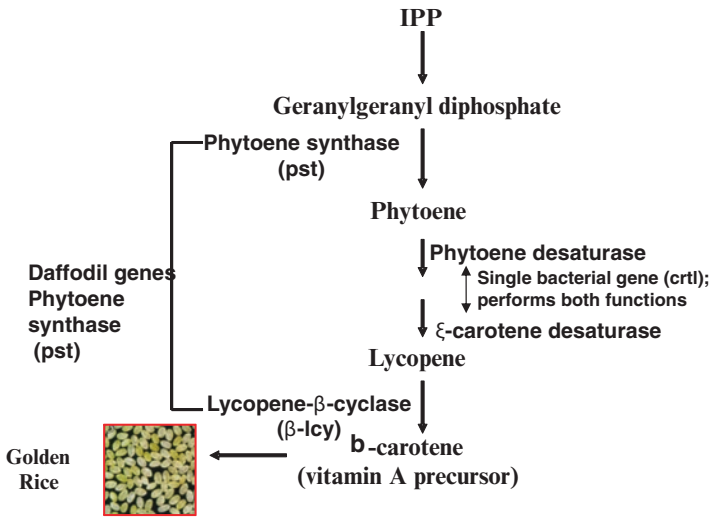

Fig. 6.7 Schematic representation of production of provitamin A (β-carotene) into rice endosperm by genetic engineering

6.4 Challenges in Plant Metabolic Engineering

Plants were never intended by the nature to be grown as crops on an industrial scale for human consumption nor were they inclined to give up their structural oligosaccharides to provide green energy. The plants were to sustain their existence as an important entity of the universe using their remarkable feat of metabolic networks and resources. The humans have, however, engineered these metabolic networks and resources for their own benefits. The technology has made great strides in plant metabolic engineering over the last two decades, with notable success stories including Golden rice. Significant challenges, however, still remain which need to be addressed and are as follows.

6.4.1 Unexplored Regulation of Secondary Metabolism

The lack of complete understanding of the regulation of secondary metabolism, especially in the complex alkaloid biosynthesis, hinders the determination of an effective metabolic engineering strategy to achieve a target metabolite production phenotype. The complexities comprise pathway compartmentalization, the existence of sometimes multiple alkaloid biosynthetic pathways and the regulatory control mechanisms.

6.4.2 Species-Specific Pathways

The progress in isolating genes involved in secondary metabolism is limited due to species specificity, the inability in producing large numbers of mutants, their intermediate precursor availability, their analysis and the instability of target compound due to environmental influence. The major bottleneck for secondary metabolism, however, will be its species specificity as only early parts of secondary metabolite pathways are common to most plant species. For example, the early steps of biosynthetic pathways leading to the synthesis of flavonoids and terpenoids are common, but the later steps are species specific. Thus, the homology among genes can only be used to develop strategies to clone genes for the earlier steps in these pathways. However, the genes encoding enzymes involved in the more specific alteration of the basic skeletons only can be studied at the level of the particular plant producing specific metabolite.

6.4.3 Cell Compartmentalization and Tissue Differentiation

The highly compartmentalized nature of enzymes, substrate precursors and metabolic intermediates contribute to the complexity of secondary metabolite production, which is regulated at a different level. Plants also have numerous specialized and differentiated organs in which physiological processes and gene expression may differ substantially and further adding onto the complexity of secondary metabolite production. These issues complicate the targeting gene strategies in plants. Moreover, if the engineered plants are going to be propagated as crops, environmental effects may add to the level of variability and unpredictability. There is increasing evidence that intra- and intercellular translocation of enzymes is one of the key elements in secondary metabolite production. Thus, localization of enzymes to diverse cellular compartments is important in protein targeting and assembly in alkaloid pathway and requires more integrated research at gene expression and regulation levels.

6.4.4 Unpredicted or Unexpected Outcome

There are several other limitations that are encountered in genetic engineering efforts to enhance secondary metabolite biosynthesis in plants. These include gene silencing, unpredictable results due to complex network of genes and no increase in concentration of desirable metabolites up to the level of commercialization. Techniques used to introduce new genes into plants also do not predict actual site of integration and the level of gene expression, even when a strong promoter is used. It is often hard to accurately guess the actual biological roles of certain enzymes

explicitly based on bioinformatics, due to their ambiguity towards substrates and the relative easiness to change substrate or product specificity by introducing minor change in sequence or structure of the enzymes.

Single-enzyme perturbation of alkaloid pathways resulted in unexpected metabolic consequences suggesting the existence of key rate-limiting steps, potential multienzyme complexes or unsuspected compartmentalization. Overexpression of COR1 (codeinone reductase), the last enzyme in morphine biosynthesis, increased the morphine and codeine contents in transgenic poppy (Larkin et al. 2007). However, thebaine, an intermediate that occurs prior to codeinone reductase in the 23 branch pathways, was also unexpectedly significantly increased. The knock down of COR1 with RNAi technology would expect to suppress the accumulation of codeinone and morphinone which is the immediate precursor of COR. The results of this experiment were, however, not as expected. The amount of morphinan alkaloids decreased, while the biosynthesis of (S)-reticuline, a seven-step early upstream metabolite, in the pathway was increased (Allen et al. 2004). The increased concentration of (*S*)-reticuline suggested a negative feedback between the morphinan pathway and benzylisoquinoline pathway. (*S*)-reticuline is the key branch point metabolite in benzylisoquinoline pathway. Studies also suggest that codeinone reductase may also serve as a control point and overexpression would lead to higher accumulation of morphinan. Thus, the complexity and redundancy of many biosynthetic pathways coupled to incomplete knowledge of their regulation could lead to an unpredictable outcome from a targeted metabolic engineering strategy.

6.5 Conclusions

Metabolic engineering of plants belongs to the category of the second generation of plant genetic engineering. It is an efficient way to genetically modify the target plant metabolites and is also a hot spot of genetic engineering of medicinal plants. Since the biosynthetic pathways in plants are extensive and complicated, therefore, they require manipulation of more than one enzyme to produce the desired end product. Also, engineering secondary metabolism in plant cells primarily aims at increasing the content of desired secondary compounds, lowering the levels of undesirable compounds and introducing novel compound production into specific plants. For these kinds of studies, metabolic pathways profiling is required which includes understanding of metabolic regulation of the desired pathways at the level of intermediates and enzymes. Such studies eventually lead to the production of transgenic plants or plant cell cultures with higher accumulation of desired secondary metabolite. Aside from practical applications of plants or plant cell cultures, the knowledge gained will be helpful in establishing the functional/adaptive roles of secondary metabolites in plants.

6.6 Future Perspectives

The next decade holds great expectations and challenges for synthetic biologists, metabolic engineers and plant breeders. The repertoire of bio-derived products will grow, and many new technologies that stimulate and control biological processes at cellular and organ level will emerge. To direct these kinds of metabolic manipulations in plants or plant cell cultures, a knowledge base by integrating information at genetic, cellular and molecular levels has to be developed by scientists. It will give a better understanding of the basic metabolic processes involved and could provide the key information needed to produce high-value metabolites.

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Chapter 7
Plastome Engineering: Basics Principles and Applications

Malik Zainul Abdin, Priyanka Soni, and Shashi Kumar

Abstract Genetic material in plants is distributed into the nucleus, plastids, and mitochondria. Plastid has a central role of carrying out photosynthesis in plant cells. Plastid transformation is an advantage to nuclear gene transformation due to higher expression of transgenes, absence of gene silencing and position effect, and transgene containment by maternal inheritance, i.e., plastid gene inheritance via seed not by pollen prevents transmission of foreign DNA to wild relatives. Thus, plastid transformation is a viable alternative to conventional nuclear transformation. Many genes encoding for industrially important proteins and vaccines, as well as genes conferring important agronomic traits, have been stably integrated and expressed in the plastid genome. Despite these advances, it remains a challenge to achieve plastid transformation in non-green tissues and recalcitrant crops regenerating via somatic embryos. In this chapter, we have summarized the basic requirements of plastid genetic engineering and discuss the current status and futuristic potential of plastid transformation.

7.1 Introduction

Genetic material in plants is divided into three organelles of the nucleus, mitochondria, and plastid. The plastid when present in green form in plant is called as chloroplast, which carries its own genome and expresses heritable traits (Ruf et al. 2001). Chloroplast's DNA, often abbreviated as ctDNA/cpDNA, is known as the plastome (genome of a plastid). Its existence was first proved in 1962 and sequenced

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in 1986 by two Japanese research teams. Since then, over hundreds of chloroplast DNAs from various plant species have been sequenced. The plastid DNA (ptDNA) of higher plants is highly polyploidy, and double-stranded circular genomes are about 120–160 kilobases. The number of plastids per cell and the number of ptDNA per plastid vary species to species. For example, an *Arabidopsis thaliana* leaf cell contains about 120 chloroplast organelles and harbors over 2000 copies of the 154 Kb size plastid genomes per cell (Zoschke et al. 2007), whereas *Nicotiana tabacum* leaf cell contains about 10–100 chloroplast organelles per cell and harbors over 10,000 copies of ptDNA per cell (Shaver et al. 2006). The photosynthetic center of the plant cells and eukaryotic algae provides the primary source of the world's food (Wang et al. 2009). Other important activities that occur in plastids include evolution of oxygen, sequestration of carbon, production of starch, and synthesis of amino acids, fatty acids, and pigments (Verma and Daniell 2007).

Transformation of the plastid genome was first accomplished in *Chlamydomonas reinhardtii*, a unicellular alga (Boynton et al. 1988), followed by plastid transformation in *N. tabacum*, a multicellular flowering plant (Svab et al. 1990; Daniell et al. 2004). Plastid transformation since has been extended to *Porphyridium*, a unicellular red algal species (Lapidot et al. 2002), and the mosses *Physcomitrella patens* (Sugiura and Sugita, 2004) and *Marchantia polymorpha* (Chiyoda et al. 2007). In higher plants, plastid transformation is reproducibly performed in *N. tabacum* (Svab and Maliga 1993), tomato (Ruf et al. 2001), soybean (Dufourmantel et al. 2004), carrot (Kumar et al. 2004a), cotton (Kumar et al. 2004b), lettuce (Lelivelt et al. 2005; Kanamoto et al. 2006), potato (Nunzia 2011), and cabbage (Liu et al. 2007; Tseng et al. 2014). Monocots as a group are still recalcitrant to plastid transformation. It is assumed that in the next few years, there may be surge in commercial applications using this environmental-friendly technology due to several advantages over conventional nuclear transformation, like gene containment and higher expression levels of foreign proteins, the feasibility of expressing multiple proteins from polycistronic mRNAs, and gene containment through the lack of pollen transmission (Kittiwongwattana et al. 2007; Wang et al. 2009). The gene transfer is maternally inherited in most of the angiosperm plant species (Hagemann 2004). To obtain a genetically stable chloroplast transgenic also known as transplastomic plant, all plastid genome copies should be uniformly transformed with foreign gene.

7.2 Tools and Elements for Chloroplast Engineering

Ruhlman et al. (2010) emphasized the role of endogenous regulatory elements and flanking sequences for an efficient expression of transgenes in chloroplasts of different plant species.

7.2.1 Promoters

An efficient gene expression level in plastid is determined by the promoter. It contains the sequences which are required for RNA polymerase binding to start transcription and regulation of transcription. In order to obtain high-level protein accumulation from expression of the transgene, the first requirement is a strong promoter to ensure high levels of mRNA. Chloroplast-specific promoters are essential to ensure an efficient accumulation of foreign protein into chloroplasts in algae and plants (Gao et al. 2012; Sharma and Sharma 2009).

Plastid transcription is regulated by the combined actions of two RNA polymerases recognizing different promoters, a T7-like single-subunit nuclear-encoded polymerase (NEP) and a bacterium-like α2ββ′ plastid-encoded polymerase (PEP). Transcription in undifferentiated plastids and in non-green tissues is primarily regulated by the NEP. The production of rRNA and of mRNAs encoding ribosomal proteins is included in the PEP regulation, which results into the accumulation of functional PEP. Many plastid promoters contain both the PEP and NEP transcription start sites (Allison et al. 1996; Hajdukiewicz et al. 1997).

The 16S ribosomal RNA promoter (Prrn) like psbA and atpA gene promoters are commonly used for chloroplast transformation. These promoters drive the high level of recombinant protein expression in plastid transformation. P*rrn* contains both PEP and NEP transcription start sites, whereas P*psbA* contains only a PEP transcription start site (Allison et al. 1996).

7.2.2 5′ UTRs

The 5′ UTR is important for translation initiation and plays a critical role in determining the translational efficiency. Transcriptional efficiency is regulated by both chloroplast-specific promoters and sequences contained within the 5′ UTR (Klein et al. 1994). Many reports have revealed that translational efficiency is a rate-limiting step for chloroplast gene expression (Eberhard et al. 2002). Thus, 5′ UTRs of plastid mRNAs are key elements for translational regulation (Nickelsen 2003), and many chloroplast genes are regulated at the posttranscriptional level (Barkan 2011). However, the nature of these internal enhancer sequences has not been studied well (Klein et al. 1994).

The most commonly used 5′ UTRs are those of the plastid *psbA* gene, *rbcL*, and the bacteriophage T7 gene 10. It has been incorporated into many chloroplast transformation vectors that give rise to extremely high levels of transgene protein expression (Kuroda and Maliga 2001a, b; Oey et al. 2009a, b; Tregoning et al. 2003; Venkatesh and Park 2012).

7.2.3 3′ UTRs

The 3′ UTR plays an important role in gene expression, and it contains the message for transcript polyadenylation that directly affects mRNA stability (Chan and Yu 1998). Plastid 3′ UTRs, cloned downstream of the stop codon, contain a hairpin-loop structure that facilitates RNA maturation and processing and prevents degradation of the RNA by ribonucleases (Stern et al. 2010). Valkov et al. (2011) reported the roles of alternative 5′ UTR and 3′ UTRs on transcript stability and translatability of plastid genes in transplastomic potato, suggesting the role of 3′ UTRs on transcript stability and accumulation in amyloplasts. Some 3′ UTRs can affect 3′-end processing and translation efficiency of transgenes expression in chloroplasts (Monde et al. 2000). 3′ UTRs like *rps16*, *rbcL*, *psbA*, and *rpl32* 3′ UTRs are being commonly used in chloroplast transformation system. The most commonly used 3′ UTR is T*psb*A (Gao et al. 2012; Kittiwongwattana et al. 2007).

7.2.4 Downstream Boxes

The downstream box (DB) containing about 10–15 codons downstream of the start codon was first identified in *E. coli* (Sprengart et al. 1996). It has major effects on accumulation of foreign protein in *E. coli*, acting synergistically with the Shine–Dalgarno sequences upstream of the start codon to regulate protein accumulation. Kuroda and Maliga (2001b) reported that sequences like the DB region in *E. coli* appeared to function in tobacco chloroplasts. Their mutational analyses revealed that the DB RNA sequence influenced the accumulation of foreign transgenic protein. Follow-up studies on the effects of the DB region on transgene regulation in chloroplast have found major changes in protein accumulation and studied using a number of different transgenes and corresponding protein products (Gray et al. 2009; Hanson et al. 2013; Kuroda and Maliga 2001a; Venkatesh and Park 2012; Ye et al. 2001).

7.2.5 Selection Marker Genes

Since ptDNA (plastid DNA) is present in many copies, selectable marker genes are critically important to achieve uniform transformation of all genome copies during an enrichment process that involves gradual sorting out non-transformed plastids on a selective medium (Kittiwongwattana et al. 2007; Maliga 2004). The first selection marker gene used in chloroplast transformation was plastid16S rRNA (*rrn16*) gene (Svab et al. 1990). The *aadA* gene encoding aminoglycoside 3-adenylyltransferase is used as a selection marker gene for genetic transformation of many plant species (Goldschmidt-Clermont 1991; Svab and Maliga 2007).

The *npt II* was also used as a selectable marker for plastid transformation in tobacco, (Carrer et al.1993). The bacterial *bar* gene, encoding phosphinothricin acetyltransferase (PAT), tested as a marker gene but resulted in extremely low transformation efficiency (Lutz et al. 2001). Another poor marker gene is the betaine aldehyde dehydrogenase (BADH) gene, which confers resistance to betaine aldehyde in tobacco (Daniell et al. 2001b; Wang et al. 2009).

The unwanted antibiotic selection marker after obtaining uniformly stable chloroplast transgenic plants can be precisely removed by Bxb1 recombinase. It is a unique molecular tool that can be used to remove unwanted antibiotic or herbicide resistance genes after genetic engineering of chloroplast DNA before releasing the plants into commercial production (Shao et al. 2014).

7.3 Methods for Chloroplast Engineering

Plastid transformation has been preferably carried either by biolistic bombardment of plant tissue with a chloroplast-specific transformation vector (Svab and Maliga 1993) or by polyethylene glycol-mediated transformation of protoplasts (Golds et al. 1993). It occurs by homologous recombination between the flanking sequencings (native chloroplast DNA) of chloroplast-specific transformation vector and the plastid genome at the predetermined site along with gene(s) of interest (Maliga 2004). After integration of transgenes flanked by homologous recombination sites into the chloroplast, repeated rounds of tissue regeneration on stringent antibiotic selection are needed to achieve the homoplasmy status (Kumar and Daniell 2004), i.e., all wild-type plastid genomes (plastomes) to be replaced with the foreign DNA cassette (Fig. 7.1). Transplastomic plant may express foreign protein of 5–15 % total soluble protein (Maliga and Bock 2011; Scotti et al. 2012) and in some reports are over of 30 % total soluble protein (Daniell et al. 2001a; De Cosa 2001; Lentz et al. 2010).

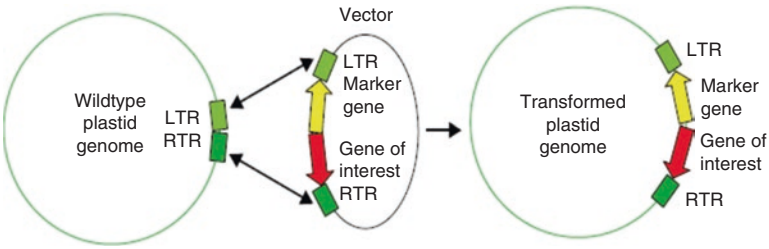

Fig. 7.1 A transformed plastid genome is formed by two recombination events that are targeted by homologous sequences. The plastid genome segments that are included in the vector are marked as the left (LTR) and right targeting regions (RTR) (after Maliga 2002, Current Opinion in Plant Biology)

Table 7.1 First reported agronomic traits via the chloroplast genome

Trait	Transgene	Promoter	5′/3′ UTRs	Homologous recombination site	References
Insect resistance	*Cry*1*A* (c)	Prrn	rbcL/Trps	trnV/rps12/7	McBride et al. (1995)
Herbicide resistance	*Aro*A	Prrn	ggagg/TpsbA	rbcL/accD	Daniell et al. (1998)
Insect resistance	*Cry*2*Aa*2	Prrn	ggagg (native)/ TpsbA	rbcL/accD	Kota et al. (1999)
Herbicide resistance	*bar*	Prrn	rbcL/accD	rbcL/accD	Iamtham and Day (2000)
Insect resistance	*Cry*2*Aa*2	Prrn	Native UTRs/ TpsbA	trnI/trnA	DeCosa et al. (2001)
Disease resistance	*MSI*-99	Prrn	ggagg/TpsbA	trnI/trnA	DeGray et al. (2001)
Drought resistance	*tps*	Prrn	ggagg/TpsbA	trnI/trnA	Lee et al. (2003)
Phytoremediation	*merAa*/*merBb*	Prrn	ggagga,b/ TpsbA	trnI/trnA	Ruiz et al. (2003)
Salt tolerance	*badh*	Prrn	ggagg/rps16	trnI/trnA	Kumar et al. (2004)
Cytoplasmic male sterility	*pha*A	Prrn	PpsbA/TpsbA	trnI/trnA	Ruiz and Daniell (2005)

The chloroplast transformation lacks the epigenetic effects and gene silencing, which may help in accumulating high levels of heritable protein (Dufourmantel et al. 2006), in contrast to nuclear transformants, where protein accumulation is quite variable among independently transformed plants (Yin et al. 2004). Moreover, plastid genomes are very rarely transmitted via pollen to non-transgenic wild-type relatives (Ruf et al. 2007). Thus, chloroplast genomes defy the laws of Mendelian inheritance in that they are maternally inherited in most species, and the pollen does not contain chloroplasts and provides a natural biocontainment of transgene flow by outcrossing. Multigene engineering is reported in a single chloroplast transformation event by introducing a six transgenes mevalonate pathway (Kumar et al. 2012) and further more number of transgenes including of artemisinic acid biosynthesis (Saxena et al. 2014). Using a single transformation event, the cry operon from *Bacillus thuringiensis* (Bt), coding for the insecticidal protein delta-endotoxin, was expressed up to 46% of the total leaf protein (DeCosa et al. 2001). Three bacterial genes coding for the polymer PHB operon were introduced in chloroplast genome (Lossl et al. 2003). Thus, foreign genes expressed in the plastid genome now provide a best system to bestow useful agronomic traits and therapeutic proteins (Daniell et al. 2005) (Table 7.1). In brief, the plastid expression system is an environmental-friendly approach (Chebolu and Daniell, 2010; Gao et al. 2012; Obembe et al. 2011).

7.4 Application of Chloroplast Engineering

Chloroplast engineering techniques have been applied in numerous fields including agriculture, industrial biotechnology, and medicine. Following are some plant traits that are improved using chloroplast engineering.

7.4.1 Insect Pest Resistance

The insect resistance genes were investigated for high-level expression from the chloroplast genome. *Cry* genes were expressed in the plastid genome, which proved to be highly toxic to herbivorous insect larvae (De Cosa et al. 2001). High-level expression (about 10 % of total soluble protein) of a *cry* gene (*Cry9Aa2*) in the plastid genome resulted in severe growth retardation of insect larvae (Chakrabarti et al. 2006). The insect-resistant transplastomic soybean plants offer an opportunity for extending this technology to food crops (Dufourmantel et al. 2005). Transgenic chloroplasts in tobacco plant conferred the resistance to the fungal pathogen *Colletotrichum* destructive (De Gray et al. 2001).

7.4.2 Abiotic Stresses

The chloroplast genetic engineering may be used for improving abiotic stress tolerance. Sigeno et al. (2009) developed the transplastomic petunia, expressing monodehydroascorbate reductase (MDAR), one of the antioxidative enzymes involved in the detoxification of the ROS under various abiotic stresses (Venkatesh and Park 2012). Craig et al. (2008) produced transplastomic tobacco plants, expressing a Delta-9 desaturase gene from wild potato species *Solanum commersonii*, to control the insertion of double bonds in fatty acid chains. It has increased the cold tolerance in transplastomic plants with altered leaf fatty acid profiles. An expression of a Delta-9 desaturase gene in potato plastids not only achieve the higher content of unsaturated fatty acids (a desirable trait for stress tolerance) but also improved the nutritional value (Gargano et al. 2003, 2005; Venkatesh and Park 2012).

Chloroplast engineering had been successfully applied for the development of plants with tolerance to salt, drought, and low temperature by overexpression of glycine betaine (GlyBet) to improve the tolerance to various abiotic stresses (Rhodes and Hanson 1993). Transplastomic carrot plants expressing BADH could be grown in the presence of high concentrations of NaCl (up to 400 mmol/L), the highest level of salt tolerance reported so far among genetically modified crop plants (Kumar et al. 2004a). To counter-affect adverse environmental conditions, many plants express the low molecular weight compounds, like sugars, alcohols, proline, and quaternary ammonium compounds (Glick and Pasternak 1998). Transplastomic

tobacco plants, expressing the yeast trehalose phosphate synthase (TPS1) gene, accumulated the trehalose thousand times higher than nuclear transgenic (Lee et al. 2003; Schiraldi et al. 2002; Venkatesh and Park 2012). Trehalose is typically accumulated under stress conditions and protects plant cells against damage caused by freezing, heat, salt, or drought stresses.

7.4.3 Herbicide Resistance

The most commonly used herbicide, glyphosate, is a broad-spectrum systemic herbicide known to inhibit the plant aromatic amino acid biosynthetic pathway by competitive inhibition of the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS), a nuclear-encoded chloroplast targeted enzyme (Bock 2007). Most of the transgenic plants resistant to glyphosate are engineered to overexpress the EPSPS gene (Ye et al. 2001); since the target of glyphosate resides within the chloroplast, engineering of plastids is an ideal strategy for developing glyphosate resistance in plants for weed control (Daniell et al. 1998; Lutz et al. 2001). The *bar* gene expression in plastid encoding the herbicide-inactivating phosphinothricin acetyltransferase (PAT) enzyme led to high-level enzyme accumulation (>7 % of TSP) and conferred field-level tolerance to glufosinate (Lutz et al.2001). The plastid engineering can provide an adequate expression of resistance genes to effectively protect the crops in the field.

7.4.4 Production of Biopharmaceuticals

A therapeutic protein, human serum albumin (HSA) was expressed in transgenic chloroplasts over 10 % of TSP, 500-fold higher than the nuclear transformation system (Millán et al. 2003). Cholera toxin B subunit (CTB) of *Vibrio cholerae*, a candidate vaccine antigen, was expressed in chloroplasts with an accumulation up to 31.1 % of TSP (Daniell et al. 2001a). Recently, chloroplast transformation in high-biomass tobacco variety Maryland Mammoth was used for expression of human immune deficiency virus type 1 (HIV-1) p24 antigen (McCabe et al. 2008). Thus, chloroplast system is most suitable for high-level expression and economical production of therapeutic proteins.

However, chloroplast organelle lacks the N- or O-glycosylation process, which is required for stability and functionality of many proteins (Faye and Daniell 2006; Wang et al. 2009). Therefore, more studies are needed for glycoprotein expression and to introduce the mechanism of glycosylation in the chloroplasts (Wang et al. 2009). Chloroplasts can be an excellent biofactory for producing the non-glycosylated biopharmaceutical proteins. A non-protein drug artemisinin biosynthesized (∼0.8 mg/g dry weight) in tobacco at clinically meaningful levels in tobacco by engineering two metabolic pathways targeted to three different

cellular compartments (chloroplast, nucleus, and mitochondria). Such novel compartmentalized synthetic biology approaches should facilitate low-cost production and delivery of drugs through metabolic engineering of edible plants (Malhotra et al. 2016).

7.4.5 Edible Vaccine

To create an edible vaccine, selected desired genes can be engineered in chloroplast to produce the encoded proteins. An edible vaccine may be composed of antigenic proteins, devoid of pathogenic genes. Plastids can be used as a green factory for producing vaccine antigens (Daniell et al. 2006; Fernandez et al. 2003; Koya et al. 2005; Tregoning et al. 2004; Watson et al. 2004). The significance of using plants to produce biopharmaceuticals may reduce the overall production and delivery costs, without any risk of therapeutic product contaminated with human pathogens (Bock 2007).

The candidate subunit vaccine against *Clostridium tetani*, causing tetanus, was expressed in tobacco chloroplast, antigen proved to be immunologically active in animal model (Tregoning et al. 2004). A nontoxic protein fragment C of the tetanus toxin (TetC) was expressed at high levels about 30 % of TSP. In another study, chloroplasts are used to produce antibiotics against pneumonia *Streptococcus pneumonia* up to 30 % of the plant's TSP, which has efficiently killed the pathogenic strains of *Streptococcus pneumoniae*. Thus, it provided a promising strategy for producing antibiotics in plants against pneumonia-causing agent.

7.4.6 Biofortification

Carotenoids are essential pigments of the photosynthetic machinery as well as important nutrition for human diet as a vitamin A precursor and β-carotene (Apel and Bock 2009). The carotenoid biosynthetic pathway localized in the plastid has been conceptualized for overexpression of a single or combination of two or three bacterial genes, CrtB, CrtI, and CrtY, encoding phytoene synthase, phytoene desaturase, and lycopene β-cyclase, respectively, to enhance the carotenoid biosynthesis in crop plants (Lopez et al. 2008; Wurbs et al. 2007). Wurbs et al. (2007) demonstrated the feasibility of engineering nutritionally important biochemical pathways in transplastomic tomato, expressing bacterial lycopene β-cyclase gene, which resulted in the conversion of lycopene to β-carotene with fourfold enhanced β-carotene content. Similarly, Apel and Bock (2009) produced the transplastomic tomato fruits expressing the lycopene β-cyclase genes from the *Eubacterium* (*Erwinia herbicola*).

Plastid engineering holds great promise for manipulation of fatty acid biosynthesis pathway genes (Rogalski and Carrer 2011) for improving food quality. Madoka

et al. (2002) replaced the promoter of the accD operon with a plastid rRNA operon promoter (rrn), which enhanced the total ACCase levels in plastids. These transformants have twofold more leaf longevity and double the fatty acid production. Transplastomic tobacco plants expressing the exogenous Delta-9 desaturase genes have increased the unsaturation level in both leaves and seeds (Craig et al. 2008). Plastid engineering can efficiently synthesize the unusual fatty acids, like very-long-chain polyunsaturated fatty acids (VLCPUFAs) by expression of four genes (three subunits ORF A, B, C of the polyketide synthase system and the enzyme phosphor pantetheinyl transferase), which are absent from plant foods (Rogalski and Carrer 2011).

7.4.7 Biopolymer Production

The production of biodegradable polymers via transgenic technology is a great challenge for plant biotechnologists (Huhns et al. 2009; Neumann et al. 2005). A number of genes encoding synthesis of biodegradable polyester have been expressed in tobacco chloroplasts (Arai et al. 2004; Lossl et al. 2003). Recently, Bohmert-Tatarev et al. (2011) reported the PHB expression up to 18.8 % dry weight of leaf tissue by improving the codons and GC content, similar to the tobacco plastome. The other targets for expressing in chloroplast may be collagen and spider silk-elastin fusion proteins, which are immensely important for biomedical application (Scheller and Conrad 2005). Guda et al. (2000) has expressed the bioelastic protein-based polymers by integration and expression of the biopolymer gene (EG121). However, its commercial production and its adequate purities remain a challenge from plant chloroplasts. Recently, Xia et al. (2010) expressed spider dragline silk by overcoming the difficulties caused by its glycine-rich characteristics, which provided a new insight for optimal expression and synthesis of plastid-targeted silk proteins in plant systems (Venkatesh and Park 2012).

7.4.8 Cytoplasmic Male Sterility (CMS)

CMS is important to produce the hybrid seed in agronomic crops. The high levels of accumulation of polyhydroxybutyrate (PHB) in tobacco resulted in male sterility and growth retardation when metabolic pathway for PHB using the three genes, phaA, phaB, and phaC, was engineered in chloroplasts (Lossl et al. 2005). Further, Ruiz and Daniell (2005) revealed that the b-keto thiolase enzyme coded by phbA gene when expressed in tobacco chloroplast was yielded 100 % male sterile plants, which might provide advantage in hybrid seed production. However, more research on inducing cytoplasmic sterility through plastid genome engineering is needed in future.

7.4.9 Quality Improvement

Chloroplast genome engineering has also been attempted to engineer nutritionally important metabolic pathways, especially for enhancement of essential amino acid biosynthesis, vitamin content, and fatty acid quality in seeds (Rogalski and Carrer 2011). Overexpression of b-subunit of the two units (a and b) of anthranilate synthase in tobacco plastids exhibited tenfold increase of free tryptophan in the leaves (Zhang et al. 2001). Plastid expression of astaxanthin, a pigment of human health (Hasunuma et al. 2008) and carotenoids (pro-vitamin A) has raised the hope of metabolic engineering of nutraceuticals in transplastomic plants. Plastid transformation can be used for producing very-long-chain polyunsaturated fatty acids, which are usually found in cold-water fishes and have potential health benefits (Bansal and Dipnarayan 2012; Rogalski and Carrer 2011).

7.5 Conclusion and Future Prospects

Up to date, many transgenes have been successfully introduced and expressed into the plastid genome of model plant tobacco and many other agronomically important crops. Still there are many important cereals crops in which plastid engineering has not yet been standardized. Plastid transformation provides high levels of transgene expression and could be used for production of proteinaceous pharmaceuticals, such as antigens, antibodies, and antimicrobials in a cost-effective manner. The routine use of plastid engineering in plant biotechnology is still a long way to go. However, there is no doubt that plastid engineering holds a great potential in the future despite of many challenges that need to be addressed before its widespread adoption, like protein purification and expression level control. Unlike other techniques, such as bacterial expression and nuclear genetic engineering or plants, chloroplast modification has succeeded in producing therapeutic proteins and vaccines at commercially feasible levels. In addition, genetically engineering the chloroplast is environmental friendly, and transgenes are contained within the plant. However, more basic research is required before chloroplast genetic engineering can be applied commercially. This includes modifying more number of agronomically important crops and vegetables and ensuring the functionality of the resultant therapeutic proteins in humans.

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Chapter 8
Genetic Engineering to Improve Biotic Stress Tolerance in Plants

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Abstract Genetic engineering of plants for resistance is an effective method to counter pathogens and pests owing to the specificity and efficiency of the technology. The genes that have been used to genetically engineer resistance are as diverse as the diseases they act against. In cases where gene-for-gene resistance coded by resistance (*R*) genes exists, engineering resistance in plants becomes a straight path. Different classes of *R* genes have been engineered to provide resistance against viruses, bacteria, filamentous phytopathogens, and nematodes. Where the resistance mechanism is not *R* gene mediated, myriad of other mechanisms have been tried. These include the use of genes coding for antimicrobial compounds against bacterial and filamentous pathogens. The cloning of transcription factors, receptor genes, proteases, and genes involved in the systemic acquired resistance (SAR) has also been found to be effective. RNA silencing against specific genes involved in pathogenicity has proved to be an efficacious strategy against viruses and nematodes. Posttranscriptional silencing of genes coding for viral coat proteins has been successful, both scientifically and commercially. The most extensively used technology till date has been the introduction of *cry* genes from the bacterium *Bacillus thuringiensis* into plants to render them resistant against insect pests. Advances in molecular biology have paved the way for new strategies, the phenomenon of host-induced gene silencing (HIGS) being an interesting example. Amidst all the hue and cry raised against genetic modification of crops, it is necessary to highlight the scientific principles involved so as to make full use of a technology that could very well solve the problem of food shortage.

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8.1 Introduction

Plants and phytopathogens are crossed in an everlasting battle for survival. When human settlement happened and it was discovered that crop plants can be cultivated, the phenomenon of agriculture came into being. The battle that humans were previously blissfully unaware of now began affecting them. The pathogens that infect plants and methods to control them came about to be a problem never to be underestimated. Plant diseases damage the quantity and quality of crops. Their control and management burns a big hole through the pocket of agricultural economy. For a long time, disease control has been looked at with a "prevention is better than cure" point of view. Cultural methods like crop rotation, sanitation, and eradication of alternate hosts fall under this category. Even when complete eradication of the pathogen is thought of, chemical agents like pesticides and fungicides are predominantly used. Now imagine a scenario where none of these efforts are required. An agricultural utopia where you reap exactly what you sow! For this scenario to occur, complete disease resistance is the ultimate goal. Thus begins the search for natural sources of resistance. Conventional plant breeding techniques have been able to mine and harbor various natural sources of resistance. These techniques have been well established and are noncontroversial. In many cases however, the sources of resistance are not available, and even when available, not durable. Further, the pathogens that infect the plants develop mechanisms to overcome resistance. So arose the need for a technology that specifically addresses these problems without affecting the normal functions in a system as complex as life. This is where the story of genetic modification of crop plants for resistance begins. In fact cloning for disease resistance has brought about the most commercially used varieties of transgenic plants. For example, *B. thuringinesis* (Bt) crops with insect resistance (Tabashnik et al. 2013) and papaya plants with resistance to the ringspot virus (Manshardt and Drew 1998) are well-known initiatives.

This chapter describes the various attempts and trials that have been made in order to enhance resistance against various pathogens and pests in different plants. Filamentous phytopathogens including fungi and oomycetes, bacterial pathogens, viruses, nematodes, and insect pests are the five groups of organisms that have been included in this chapter. The different organisms that affect plants and strategies used against them have been addressed separately, even though some strategies are common to two or more groups of organisms.

8.2 Viruses

Viruses are infectious particles that need to and can only survive and multiply inside living host cells, making them obligate parasites. They enter the plant cell through a cut or wound on the surface and depend primarily on agents such as nematodes and insects for dissemination. The rapid spread of viral agents makes the control of

viral diseases tedious. Prevention of the pathogen from coming in contact with the host is one way of effectively controlling the advent of the disease. This strategy involves the use of virus-free seeds and the spraying of pesticides to control the agents of dissemination. But this strategy obviously fails, if the pathogen somehow is enabled access to the plant. Therefore, stronger levels of resistance are required, the best of which would be to render the hosts themselves resistant by means of genetic engineering. This strategy requires the use of genes that, in natural sources, are known to provide resistance against viruses. The development of transgenic crops against viruses has been successfully employed since mid-1980s. Enhancing resistance to viruses has been the most successful when compared to other pathogens.

The weapons in the plant's armory against viruses can be divided into two types: the *R* genes or resistance genes and the RNA silencing pathway. The *R* genes are involved in specific defense responses against a variety of pathogens including viruses. The *R* gene products (R proteins) directly or indirectly interact with the components of a viral pathogen and mediate defense responses. One example is a transcription factor, TCV-interacting protein (TIP) in *Arabidopsis thaliana* that directly interacts with the coat protein of turnip crinkle virus (TCV) (Ren et al. 2000). The downstream effect of *R* gene activation can be varied ranging from hypersensitive response (HR) to systemic acquired resistance SAR.

RNA silencing is the mechanism which uses dsRNA (double-stranded RNA) to recognize and subsequently degrade homologous sequences of RNA. The key players of this drama are a dsRNA trigger, DICER-like enzymes that catalyze the cleavage of dsRNA into small RNAs, the processed product which can be either siRNA (small interfering RNA) or miRNA (microRNA), and the RISC complex which then uses these cleaved RNAs to recognize homologous sequences and destroy them. RNA silencing plays a role in various developmental aspects of a plant's life, but here we are interested in its role in natural immunity of plants against viruses. The *R* gene-mediated resistance and the RNA silencing pathway have been extensively employed to enhance plant resistance against viruses along with some other novel aspects that have been tried by adventurous scientists.

The *R* genes are the class of plant genes that are most studiously analyzed and used when it comes to genetically engineered resistance. Resistant genes against viruses can be either dominant or recessive in nature. Many *R* genes discovered till date have been found to code for monogenic dominant resistance, and this is true to a large extent in the case of viral pathogens also. Several *R* genes discovered in case of viral immunity have been shown to belong to the NBS-LRR type, but their products lack a transmembrane domain which is not surprising when the intracellular lifestyle of viruses is taken into consideration. Tobacco mosaic virus (TMV) is a pathogen of tobacco plants against which there exist various control practices. The *N* gene is an NBS-LRR-type *R* gene of tobacco that had been isolated by transposon tagging. Following the tagging, the genomic DNA fragments containing the *R* gene were shown to impart resistance against TMV to TMV-susceptible tobacco plants making it the first *R* gene to be cloned for promoting resistance against viruses (Whitham et al. 1994). The use of other *R* genes soon followed. The tomato mosaic

virus (ToMV) is another virus that is related to TMV. The *Tm-2²* is a resistance locus against ToMV in tomato. Susceptible crops, when transformed with *Tm-2²* gene, were rendered resistant to ToMV (Lanfermeijer et al. 2003). The locus *Rx* is one in potato which is known to confer resistance against potato virus X (PVX) (Bendahmane et al. 1997). The *Rx* gene product recognizes a virus coat protein and arrests the growth of the viruses at an initial stage by a process that is not associated with cell death by hypersensitive response. Later when the *Rx* of potato was cloned and expressed in potato and *Nicotiana*, extreme resistance was achieved in both the crops (Bendahmane et al. 1999). *HRT* is an *R* gene of *Arabidopsis* which shows homology to the *RPP8* gene that is involved in resistance against the oomycete, *Peronospora parasitica*. The cloning of *HRT* in tobacco plants resulted in a strange phenomenon where only 10 % of the plants showed resistance while the remaining 90 % showed HR response but still remained susceptible (Cooley et al. 2000). A subsequent experiment showed the presence of another gene in this scenario called *RRT*, the recessive allele of which acts in tandem with *HRT* to mediate resistance (Kachroo et al. 2000). The *RCY1* in *Arabidopsis* is another *RPP8*/*HRT* family *R* gene. The *RCY1* from an ecotype C24 was cloned in the susceptible variety Wassilevskija and the resulting transgenic plants were shown to be capable of effectively restricting the spread of the virus (Takahashi et al. 2002).

The successful infection of a virus and its spread inside the host depends on many host factors. Mutation of some of these genes can confer resistance against viral infection. Viral infection depends on the eukaryotic translation machinery since they lack one of their own. Genes of the translation machinery have been proved to be important in viral infection especially those of the genus *Potyvirus*. The eukaryotic translation initiation factor 4E (eIF4E) is an important host gene required for viral infection. Transposon-induced or ethyl methanesulfonate-induced eIF4E mutants of *Arabidopsis* have been shown to be resistant against viral pathogens of the *Potyvirus* genus like lettuce mosaic virus (LMV) and tobacco etch virus (TEV) (Duprat et al. 2002; Lellis et al. 2002). Following this, when some naturally occurring resistance sources were characterized at a molecular level, eIF4E was found to be an important player. The *Pvr6* in pepper is a mutant of the 4E factor whose role has been characterized in resistance against pepper veinal mottle virus (PVMV) (Ruffel et al. 2006). Further analysis in tomato has also emphasized the role of 4E in virus resistance. A mutant of 4E that is impaired in splicing was shown to be more resistant to potato virus Y (PVY) and PVMV (Piron et al. 2010).

Viruses, since they are unable to thrive independent of the host cell, requires a living vector for transmission. The use of genes that can prevent the attack of vectors that carry potential pathogens is an interesting and efficient method of control of viral diseases. The aphid *Macrosiphum euphorbiae* is an agent of various viral pathogens of potato. A resistant gene in potato called *Mi* was shown to be potent in protecting the host plant against this aphid as well as the root knot nematode (Rossi et al. 1998). An *R* gene named *Nr* in lettuce is similar to *Mi* and confers resistance against the aphid *Nasonovia ribisnigri*. The *Vat* gene in melons is an NBS-LRR-type *R* gene that controls the infestation by the aphid *Aphis gossypii* (Pauquet et al. 2004). Likewise resistance to the aphid *Acyrthosiphon kondoi* in *Medicago*

truncatula has been shown to be mapped to a region containing NBS-LRR-type *R* genes (Klingler et al. 2005).

The use of pathogen-specific molecules for the control of human diseases is an idea that dates back to the time when vaccines were developed to render people resistant against diseases. Vaccines have been used extensively for viral diseases in humans. The same phenomenon has been extrapolated to agriculture for control of viral diseases with the use of several viral proteins to develop transgenic plants. A gene from a virus or a part of it is cloned in a host plant and it somehow interferes with the life cycle of the virus. The first gene ever to be used was that of a one which coded for the viral coat protein. Tobacco cells expressing a cloned cDNA expressing the coat protein of TMV showed enhanced resistance to the virus (Abel et al. 1986). The same was very soon repeated in tomato again with the coat protein from TMV (Nelson et al. 1987). Soon after, transgenic tobacco plants which expressed a coat protein from TMV was shown to be resistant against five other tobamoviruses increasing the possibility of using viral proteins to mediate resistance (Nejidat and Beachy 1990). Two varieties of transgenic summer squash (*Cucurbita pepo spp. ovifera* var. *ovifera*) were subsequently developed named ZW-20 and CZW-3 (Arce-Ochoa et al. 1995; Clough and Hamm 1995; Fuchs and Gonsalves 1995; Klas et al. 2006). The ZW-20 expresses the coat protein of zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus (WMV). The variety CZW-3 expresses the coat protein of cucumber mosaic virus (CMV), ZYMV, and WMV and is resistant to them. These commercial varieties have been released successfully and have been durable for almost two decades. Another successful attempt at cloning coat protein was made in papaya. Papaya ringspot virus (PRSV) has been a threat to papaya growing for a long time and efforts have been undertaken to control (Gonsalves 1998). Subsequently the coat protein of PRSV was successfully incorporated into the susceptible varieties followed by field trials. Two transgenic varieties termed Sunup and Rainbow were released which were resistant to PRSV (Manshardt and Drew 1998). The use of the transgenic varieties incidentally reduced the occurrence of the disease considerably, enabling the production of non-transgenic varieties of papaya in Hawaii, the region where it was first introduced. The use of coat protein for developing resistance has been used to such an extent that a new term has been coined for this phenomenon – coat protein-mediated resistance (CPMR).

The use of virus derived proteins have been used for enhancing resistance for long without having an understanding of the process by which the resistance is brought about. When pathogen-derived resistance (PDR) was employed, in some cases it was noticed that the level of protein expression of the gene does not equate with the resistance followed by the discovery that the viral RNA, and not the viral protein, is required for mediating resistance. This opened the arena to a new phenomenon of inducing resistance known as the RNA-mediated virus resistance (RMVR). RNA silencing is a natural mechanism of resistance to viruses where pathogen-derived dsRNA is targeted processed into viral small interfering RNA (vsiRNAs). These are then loaded into the RISC complex and inhibit viral replication by targeting the RNA which has sequence similarity to the vsiRNA. This is now known as sense RNA-induced PTGS. One drawback of using this technique is that

some viruses have developed mechanisms to overcome this type of PTGS-mediated resistance. Thus genetic engineering and incorporation into plants of artificial miRNAs was suggested. Artificial miRNAs (amiRNA) are similar in structure to endogenous miRNAs. miRNAs can be artificially designed to target any gene sequence, making it a highly efficient means of PTGS. Artificial miRNAs are mostly used to target the silencing suppressors in viruses which counteract the natural RNA silencing mediated immunity of plants against viruses. Modified *A. thaliana* miRNA159 was generated to target two silencing suppressors, p69 of TMYV (turnip yellow mosaic virus) and HC-pro of turnip mosaic virus. *A. thaliana* plants expressing these miRNAs are resistant to these two viruses (Niu et al. 2006). The efficiency of a system of amiRNA depends not only on the quality of the amiRNA generated but also on some secondary structures present on the target mRNA. Since it is difficult to predict what site impedes cleavage by RNA silencing complexes, efficiency can be increased by targeting those sequences on a target mRNA that can in some ways increase its chance of getting cleaved. With this in mind, artificial that target putative RISC accessible target sites were generated. The study showed that this type of amiRNA resulted in higher degrees of resistance in *Arabidopsis* against cucumber mosaic virus (CMV) (Duan et al. 2008). The silencing suppressor HC-pro of PVY and the TGBpi/p25 was mimicked later on by using the backbones of *Arabidopsis* miR159A, miR167b, and miR171a. Transgenic *Nicotiana tabacum* plants expressing these miRNA were tested for resistance against PVX and PVY and were found to be positive (Ai et al. 2011).

Growing concerns of biosafety in transgenic plants raised the possibility of using a transient system of RNA silencing that is capable of delivering silencing molecules directly into the host. DsRNA synthesized from PMMoV (pepper mild mottle virus), TEV (tobacco etch virus), and AMV (alfalfa mosaic virus) was exogenously applied to *Nicotiana benthamiana* plants using an *Agrobacterium tumefaciens*-mediated transient system. This led to successful interruption of infection of the plant by the previously mentioned viruses (Tenllado and Díaz-Ruíz 2001). A bacterial spray system was developed to transiently apply antiviral particles onto a plant cell. DNA fragments of the coat protein of SCMV (sugarcane mosaic virus) were amplified and cloned in *E. coli* HT115 strain. Crude extracts were obtained of the bacteria and was used to spray inoculate maize plants for successfully imparting resistance against the virus (Gan et al. 2010). The same kind of a spray system was used to deliver RNA silencing molecules against tobacco mosaic virus (TMV) in tobacco (Sun et al. 2010). One major drawback of this system is the lack of heritability of resistance which would result in a need for continuous spraying for sustenance of resistance.

A different approach to control viral plant diseases is the expression of antibodies against viral proteins in plants. Known as plantibodies, this has successfully been used to impart resistance in a variety of crops. Antibodies against artichoke mottled crinkle virus (AMCV) were raised and cloned in *N. benthamiana*. The transgenic plants raised showed lower accumulation of the virus (Tavladoraki et al. 1993). *N. benthamiana* was further subjected to a second attempt at a similar

approach with antibodies against the coat protein of beet necrotic yellow vein virus (Fecker et al. 1996).

Some other isolated attempts have also been made in order to impart resistance to viruses, some of which are being discussed: the use of plant protease inhibitors, the use of ribosomal inactivating proteins, and the use of interferon-like systems, replicases, and movement proteins. Viruses require the use of cysteine proteases to cleave some of their own polyproteins for successful infection. The cysteine protease inhibitor oryzacystatin was cloned in tobacco leading to successful resistance of the transgenic plants against tobacco etch virus (TEV) and PVY. When tested against TMV, no resistance was observed which is not surprising since TMV does require the processing polyproteins by cysteine proteases (Gutierrez-Campos et al. 1999). Antiviral proteins known as ribosome-inactivating proteins (RIPs) are present in some plants which inactivate translation by removing an adenine from 28 s rRNA. They are targeted specifically to vacuoles thus ensuring their separation from the endogenous 28 s rRNA. An RIP from pokeweed called PAP was cloned in *N. benthamiana*, conferring broad-spectrum resistance to several viruses (Lodge et al. 1993). Virus infection in higher vertebrates is counteracted partly by an RNA degradation system using interferons. Though counterparts of interferons have not been reported in plants, human members have been used in an attempt to raise transgenic tobacco plants resistant against TEV, TMV, and AMV (Mitra et al. 1996). Replicase is a gene that as its name suggests propagates the replication of viruses. The *Rep* gene of tobacco was the first used in this class for developing transgenic plants resistant to TMV (Golemboski et al. 1990). The same was very soon employed in other cases like early browning virus (EBV) of pea, PVY, and CMV (MacFarlane and Davies 1992; Audy et al. 1994; Hellwald and Palukaitis 1995). Cell-to-cell movement of viruses is mediated by a set of proteins known as the movement proteins (MP) which in tobamoviruses are known to change the gating property of the plant plasmodesmata to enable virus infection. Transgenic tobacco plants expressing a modified MP protein was rendered resistant to TMV. This resistant was shown to occur because the modified version of the MP that is expressed in plants competes with the MP of the infecting virus (Malyshenko et al. 1993; Lapidot et al. 1993).

Resistance against viruses is one field where the use of transgenic crops have not just been attempted but successfully and durably employed in the field for at least two decades. From the start with the cloning of coat proteins to the recent attempts at modifying the RNA silencing pathway, this field has both broadened and sharpened. While different strategies have been used, each has its own advantages and disadvantages. The use of pathogen-derived resistance here deserves special mention because the genes addressed here are those that are necessary for the pathogens exclusively and therefore they pose very less threat to the environment. This must have contributed to the easy acceptance of transgenic crops of this kind.

8.3 Bacteria

Bacterial diseases wreak havoc in a wide variety of crop plants ranging from cereals to fruits and vegetables. The pathogen-associated molecular patterns (PAMPs) of bacterial pathogens are recognized by pattern recognition receptors (PRRs). Among the PAMPs, the flagellin peptide Flg22 and elongation factor EfTu have been well characterized. Flg22 is recognized by a leucine-rich repeat receptor kinase on the surface of the plant cell called FLS2 which activates a signaling cascade involving mitogen-activated protein kinases (MAPKs) and mount pattern-triggered immunity (PTI) (Asai et al. 2002). Bacteria also produce effectors to counteract PTI. Among the wide array of effectors some are known as avirulence genes or factors (*Avr*) which interact with resistance genes (*R* genes) of the plant in what is known as the gene-for-gene interaction. Bacteria employ secretion systems to release effectors into the host cell. Type II is involved in the cause of soft rot by *Erwinia* and releases cell wall-degrading enzymes. Type IV on the other hand is required for the secretion of proteins and DNA of *Agrobacterium.* Type III (T3SS) is of cardinal importance in that it ensures the release of effector proteins directly into the plant cell.

A variety of methods are undertaken for the control of bacterial crop diseases. The use of agrochemicals, crop rotation, and the control of the pests that harbor the pathogens are some that make the list. However, these conventional methods fail in some cases. Moreover, they are more focused on prevention of the disease rather than its control. So, the use of natural sources of resistance to increase the resistance of plants might be a better idea, one that is already being used in classical breeding. The extension of the same in genetic engineering will be beneficial in controlling diseases. The sources of resistance that are generally used for engineering resistance of plants against bacteria are *R* genes and other defense-related genes, antibacterial proteins like lysozyme and magainin, and transcription factors.

As early as 1993, Noel Keen and his colleagues put forth an idea that cloning *R* genes might be a useful strategy for improving crop resistance. In their words "The incorporation of resistance genes into agronomically important crop plants is the most economically effective method for controlling plant disease. This biological disease control strategy is heritable and, therefore, inexpensive and permanently available once introduced" (Keen et al. 1993). The idea was very soon put into practice with *Pto*, an *R* gene of tomato that is known for resistance against *Pseudomonas syringae* pv. *tomato*. The *Pto* region was identified in a yeast artificial chromosome (YAC) clone and was used to probe a cDNA library. The cDNA clone that represented the *Pto* family, when cloned into susceptible tomato plants, made them resistant (Martin et al. 1993). The *Pto* gene was characterized to be a kinase. This was followed by the cloning and characterization of a number of *R* genes involved in resistance against bacterial diseases, *Rps2* of *Arabidopsis* and *Xa21* of rice among many. The *Xa21* locus in rice confers resistance to different races of the pathogen *Xanthomonas oryzae* pv. *oryzae* (Khush et al. 1990). Cloning and sequencing of this locus identified a gene that was found to have a leucine-rich repeat (LRR) motif and

a serine threonine kinase-like domain (Song et al. 1995). Thereafter, *Xa21* was successfully cloned into rice susceptible to bacterial blight caused by *X. oryzae* pv. *oryzae*, making it resistant (Wang et al. 1996). The *Xa21* gene was successfully transformed into sweet orange (*Citrus sinensis*) rendering them resistant to citrus canker disease caused by *Xanthomonas axonopodis* pv. *citri* (Mendes et al. 2010). In the *Xanthomonas* genus another bacteria causes the bacterial streak disease *X. oryzae* pv. *oryzicola. Rxo1*, resistant gene of maize which is involved in resistance against an unrelated pathogen *Burkholderia andropogonis*, when cloned in rice proved to be effective in resistance against bacterial streak (Zhao et al. 2005). This nonhost transfer of *R* genes between species opens new prospects in the use of *R* genes for controlling bacterial diseases.

In cases where a gene-for-gene resistance coded by an *R* gene is not available, the genes involved in SAR, especially *NPR1* (non-expressor of PR1), have been used to genetically engineer plants for resistance against bacteria. Overexpression of *NPR1* in *Arabidopsis* enhanced resistance against *P. syringae* and also an oomycete *Peronospora parasitica* (Cao et al. 1998). The same phenomenon has been extended to crop plants. *Arabidopsis NPR1* was overexpressed in rice and the transformed plants were subjected to the bacterial blight pathogen *X. oryzae* pv. *oryzae.* Resistance was enhanced, making it the first attempt at cloning an *NPR1* gene of *Arabidopsis* in a monocot (Chern et al. 2001). In another attempt, tomato plants expressing an *Arabidopsis NPR1* gene displayed an increased resistance toward a variety of pathogens including those that cause bacterial wilt (*Ralstonia spp.*) and bacterial spot (*Xanthomonas spp.*) (Lin et al. 2004).

Of all the modes of resistance that plants employ against pathogens, there is a curious one, the production of antimicrobial agents that can be either proteins or metabolites. The induction of these can be at the site of infection or at a point far away. The antimicrobial agents that have been used to engineer crop resistance are varied. Most of the antimicrobial agents used have been derived from a non-plant source. Two of these, attacin and cecropin, are derived from the giant silk moth *Hyalophora cecropia*. The cloning of *attacin E*, a lytic peptide in apple, enhanced the crop's resistance against *Erwinia amylovora* (Norelli et al. 1994). The same was further confirmed by the stable expression of *attacin E* in orchard grown apple trees, and their resistance against *E. amylovora* was studied over a period of 12 years (Borejsza-Wysocka et al. 2010). Cecropin B is another lytic peptide isolated from the giant silk moth *H. cecropia* that are known to possess antimicrobial properties against gram-positive and gram-negative bacteria. The idea of using cecropins for improving resistance was used as far back as 1994 where tobacco plants expressing cecropin mRNA and protein were checked for resistance against *P. syringae* pv. *tabaci.* The transformed plants did not show a drastically reduced disease resistance (Hightower et al. 1994). This was later attributed to be due to the degradation of cecropin by plant proteinases (Mills et al. 1994). In an attempt to counteract the cellular degradation of cecropin B, it was fused with the secretory peptide sequence of barley alpha amylase gene and tomato plants were transformed with this construct. The secretory sequence here increases the chance of secretion of the desired gene. Surprisingly, these transgenic tomato plants were rendered resistant to *Ralstonia*

solanacearum and *Xanthomonas campestris* (Jan et al. 2010). Cecropin A, another cecropin, was expressed in the yeast *Pichia pastoris* and its effect on the postharvest blue mold disease on apple was evaluated. These yeasts were found to reduce the number of spores in vitro and inhibit the development of *Penicillium expansum* that causes apple blue mold (Ren et al. 2012).

Magainin is another antimicrobial peptide, one which is derived from the African clawed frog *Xenopus laevis.* The antimicrobial activity of magainin is so efficient that the time it takes to kill the bacteria is faster than their doubling time, reducing considerably the chances of emergence of bacterial resistance (Hancock 1997). The toxicity of magainins are selective to prokaryotes over eukaryotes which makes the potential use of this peptide for engineering crops for disease resistance a good idea since there is no danger of it being toxic to humans. In another attempt of using magainin-like peptides, a brilliant idea of using the chloroplast genome for transformation was employed. The benefit of using chloroplast genome for transformation is that it ensures the compartmentalization of the peptide and enhanced release at the site of infection. Also since plastid DNA is lost during the maturation of pollen it contains the gene to be maternally inherited and prevents it from being transferred to the next generation through pollen. Thus, the incorporation of MSI-99 into the chloroplast genome was accomplished, and the transgenic plants were shown to be healthy without significantly deterring any important biological function. The transgenic plants showed enhanced resistance against infection of *P. syringae* pv. *tabaci* (DeGray et al. 2001). A modified version of magainin known as magainin-D was used in another case to successfully render potato plants resistant to *Erwinia carotovora*, the causative agent of the diseases black leg and soft rot. Lines of potato resistant to the pathogen were further characterized by tedious 3 years of pathogen assays (Barrell and Conner 2009).

Another naturally available antibacterial protein is the lysozyme which is a glycoside hydrolase enzyme that disrupts the structural features of bacterial cell wall by hydrolyzing the peptidoglycan. Different lysozymes like hen egg white lysozyme from chicken, T4 lysozyme from T4 bacteriophages, and human lysozymes have been used for cloning resistance. A fusion construct of T4 lysozyme with an alpha amylase signal peptide was transformed into potato and made to be secreted on the intercellular spaces. The transgenic plants were shown to show resistance against *E. carotovora*, the causative agent of black leg and soft rot. Since this bacterium colonizes the apoplast, the secretion of lysozyme into the intercellular spaces helps contain the spread of infection effectively (Düring et al. 1993). Human lysozyme and chicken lysozyme apart from hydrolyzing peptidoglycan, has also the added advantage of cleaving chitin. Transgenic potato plants harboring a human lysozyme gene showed enhanced resistance not only to the bacterium *P. syringae* but also the fungus *Erysiphe cichoracearum* that causes powdery mildew of potato (Nakajima et al. 1997). Lysozyme from hen egg white was modified to enhance its heat stability and was employed to transform tobacco. The resulting transformed

plants exhibited increased resistance against gram-negative bacteria (Trudel et al. 1992).

Pathogens are efficient in developing defense against various features of the plant immune system including pattern recognition receptors (PRRs) that recognize conserved features of the pathogens. Effectors are deployed to target the pattern-triggered immunity (PTI) and these are generally specific to the PRRs of the host plant. With this in mind, a hypothesis was formed to transfer PRR genes between plant families and thus enhance resistance. The *EFR* gene of cruciferous plant *A. thaliana* was transferred to solanaceous plants, *N. benthamiana* and *Solanum lycopersicum.* The resulting transgenic plants were rendered resistant to a variety of pathogenic bacteria like strains of *Pseudomonas*, *Xanthomonas*, and *Ralstonia* (Lacombe et al. 2011).

The phytohormone ethylene controls various aspects of a plant's life including response to stress, biotic and abiotic. The genes that are induced by ethylene in response to pathogen attack belong to different classes, one of them being the *PR* (pathogenesis-related) protein genes. The promoters of some *PR* genes flaunt an 11 bp sequence known as the GCC box which has been known to be the target of ethylene-responsive element-binding proteins (ERBPs). An ERBP transcription factor that contains an AP2-like domain, when overexpressed in tobacco plants, resulted in an improved salt tolerance and disease resistance (Park et al. 2001). The story of ERBP transcription factors in disease resistance does not end there. In tomato there is an ERBP factor Pti5 that is known to interact with Pto, the product of a *R* gene. The overexpression in tomato of a translational fusion product of *Pti5* with *VP16*, which encodes for an activation domain, brought up considerable resistance against *P. syringae* pv. *tomato* (He et al. 2001). The importance of this family of transcription factors was further proved in tobacco. A cotton gene *GbERF* was overexpressed in tobacco in an interspecific gene transfer leading to enhanced production of *PR* genes and resistance against *P. syringae* pv. *tabaci* (Qin et al. 2006).

Engineering resistance to bacterial pathogens has predictably used strategies that involve the manipulation of plant defense genes like it was the case with the filamentous phytopathogens. But in this case one important difference is that bacteria are prokaryotic organisms. Hence specific antibacterial molecules have also been used which is beneficial from a practical point of view. The use of prokaryote-specific proteins and peptides poses minimal threat to the human and animal consumers and thus reduces the issue of biosafety.

8.4 Filamentous Phytopathogens

Filamentous phytopathogens, a term that includes a diverse group of organisms, pose a massive threat to crops, causing some of the most devastating diseases, the infamous potato late blight being one of the many. They include the fungi, majorly belonging to the subphyla *Ascomycota* and *Basidiomycota*, and the oomycetes.

Based on the mode of nutrition, these pathogens are classified into three: biotrophs, necrotrophs, and hemibiotrophs. Biotrophs are those that invade and obtain nutrition from living tissue. Necrotrophs on the other hand kill the host tissue so as to extract nutrients from the dead tissue (Glazebrook 2005). Hemibiotrophs are those that start their infection cycle as biotrophs but later on kill the host cells and establish a necrotrophic mode (Oliver and Ipcho 2004).

Pattern-triggered immunity in the case of filamentous pathogens involves the recognition of conserved molecules such as chitin, the major component of the fungal cell wall. PRRs survey the arena for such molecules, detect, and pass on the signal downstream, activating defense molecules. Fungi and oomycetes release molecules known as effectors which can surpass PTI and make plants vulnerable (Jones and Dangl 2006). This is when resistance genes or *R* genes come into picture, by detecting effectors directly or indirectly and rendering them ineffective (Dangl et al. 2013). The mechanism by which R proteins recognize a target was thought to be direct for many years, in which case a classical seemingly simple gene-for-gene resistance takes place. An R protein binds to a target directly and activates signal transduction pathways leading to resistance. However, this was proved not to be universal. In some cases, an Avr protein recognizes a protein that is guarded by an R protein. The interaction of the guardee with the Avr protein is sensed by the R protein which is then activated. This is known as "guard hypothesis" (Jones and Dangl 2006). There are different classes of *R* genes, the majority of them being the NBS-LRR type that contains a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR).

Engineering resistance in plants against pathogens of the filamentous type requires the use of a variety of genes. These genes can either be those that are directly or indirectly involved in plant defense signaling or those that encode some kind of molecule that possess antifungal activity. The *R* genes have been cloned successfully for enhancement of resistance in different crop plants with satisfactory results. The late blight of potato caused by the oomycete *Phytophthora infestans* is a disease against which the host plant has been tried to be modified by a variety of methods. The wild cultivar of potato *Solanum demissum* was found to have 11 *R* genes that conferred resistance to late blight, but some races of pathogens were able to overcome this resistance. This led to the search for other sources of resistance, leading to the discovery of gene *RB* (NBS-LRR type) from *S. bulbocastanum* (Song et al. 2003). The *RB* locus was cloned in a susceptible Katahdin variety of potato and the transformed plants were found to show resistance to five isolates of *P. infestans* including the one that could overpower the 11 *R* genes form *S. demissum.* The incorporation of *RB* gene increased resistance while showing no adverse effect on crop yield whatsoever. Also, it was reported that increasing the copy numbers of the incorporated *RB* gene had a positive effect on resistance (Bradeen et al. 2009). Stem rust is a disease that affects the crop barley and causes crop loss. In a successful attempt, a variety of barley (Golden Promise) susceptible to stem rust was transformed with a resistance gene *Rpg1* and was made resistant (Horvath et al. 2003). Alfalfa, a major forage legume crop, is known to be threatened by anthracnose, a disease caused by the fungus *Colletotrichum trifolii*. This fungus is also known to

infect the model legume *Medicago truncatula*. The *RCT1* gene, an NBS-LRR type *R* gene of *M. truncatula* when transferred to alfalfa, generates resistance against anthracnose. This successful transfer of *R* genes between two species opens a new door to the enhancement of crop resistance and increases the importance of the role of model crops in raising transgenic plants (Yang et al. 2008). Stem rust of wheat caused by *Puccinia graminis* f. sp. *tritici* is a major disease of wheat against which natural sources of resistance were incorporated. Following an outbreak in 1999, a new group of races of the pathogen was identified termed *Ug99* (Singh et al. 2011). Recently two independent groups identified two loci of *R* genes, namely, *Sr33* (Periyannan et al. 2013) and *Sr35* (Saintenac et al. 2013) coding for resistance against *Ug99* races.

Many fungal diseases that affect crop plants do not flaunt a gene-for-gene resistance, where a clear cut case of *R* gene-A*vr* gene interaction is not present. In such cases, the manipulation of the genes involved in defense signaling cascades might be a good idea. SAR is a major mode of defense activated by plants against biotrophic pathogens. The *NPR1* is a gene involved in SAR that was identified first in mutant plants that showed impairment in the activation of SAR. The use of *NPR1* as a source of resistance enables the plants to express *PR* genes more and thus contribute to their resistance. Overexpression of an *NPR1* homolog in apple renders it resistant to two of its major fungal pathogens: *Venturia inaequalis* and *Gymnosporangium juniperi-virginianae* (Malnoy et al. 2007). Recently a cross-family transfer of *NPR1* from *Brassica juncea* to rice was attempted. *BjNPR1* was expressed in *indica* varieties of rice and the transformed plants demonstrated enhanced resistance to major rice pathogens (Sadumpati et al. 2013). In a cross-species attempt, *NPR1* gene from *Arabidopsis*, when cloned in wheat under the control of a maize ubiquitin promoter, rendered the plants resistant to *Fusarium* head blight (Makandar et al. 2006).

In another attempt to control *Fusarium* head blight, transgenic wheat plants that contained a *Fusarium*-specific antibody fused to an antifungal peptide were raised. An antibody derived from chicken was fused to an antifungal peptide from *Aspergillus giganteus*. This strategy provides a novel opportunity for crop engineering against pathogens that is at the same time effective and environment-friendly (Li et al. 2008).

The use of antimicrobial peptides for enhancing crop resistance is not an isolated event. Defensins are antimicrobial peptides produced by plants, invertebrates, and vertebrates with the plant defensin family being different from others in amino acid composition. Leaf spot in peanut is a disease caused by *Phaeoisariopsis personata* and *Cercospora arachidicola*. A defensin from mustard was cloned in peanut and was found to provide protection against leaf spot pathogens. The same defensin rendered tobacco insensitive to *P. para*sitica pv. *nicotiana* (Swathi Anuradha et al. 2008). Magainins, the use of which has been discussed in the case of bacterial pathogens, were also used against fungi in some cases. MSI-99 is a synthetic analog of magainin, the expression of which in potato enhanced resistance against *Aspergillus niger* (Ganapathi et al. 2007).

The plant cell wall is a fortress that keeps out many invaders, a barrier that pathogens are required to cross. Production of enzymes that degrade the plant cell wall is a strategy employed by fungal pathogens; endopolygalacturonase (endo-PG) is one of the first secreted enzymes for this purpose. The glycoprotein polyglacturonase-inhibiting protein (PGIP) on the plant cell wall is the dragon that guards the fortress by successfully inhibiting endo-PGs (Jones and Jones 1997). This justifies the use of PGIPs for raising transgenic plants resistant to filamentous phytopathogens. PGIPs from bean and fruits like pear and raspberry have been found to be effective when used for raising resistant plants. Higher accumulation of PGIP from bean in wheat increased the crop's resistance to two fungal pathogens. Digestion of PG produced by *Fusarium moniliforme* was increased and the same result was found to recur in case of infection by *Bipolaris sorokiniana* (Janni et al. 2008). *Botrytis cinerea*, the fungus that causes gray mold on tomato, has been shown to rely heavily on the cell wall-degrading activity of endo-PGs (ten Have et al. 1998). The heterologous expression of a PGIP from pear fruit in tomato reduced the disease symptoms of *Botrytis* gray mold. Even though the initial establishment of disease symptoms was not affected in transformed plants, the progress of the disease especially the area of lesion formation was considerably reduced, as is expected in case of inhibition of endo-PG activity (Powell et al. 2000). Grape (Alexandersson et al. 2011) and raspberry (Johnston et al. 1993) are two other crops where the successful reduction of disease spread through cloning of PGIP has been achieved.

Some secondary metabolites produced by plants inhibit the growth or kill invading pathogens; these include alkaloids, terpenoids, and polyphenols, the last mentioned covering flavonoids, phenols, anthocyanins, lignins, and tannins. Engineering genes involved in secondary metabolite production is ideal if successfully carried out since it provides a basic, broad-spectrum resistance. In flax, different attempts were made to enhance the production of secondary metabolites in order to increase resistance. Genes coding for chalcone synthase (*CHS*), chalcone isomerase (*CHI*), and dihydroflavonol reductase (*DFR*) (all three are enzymes required for flavonoid biosynthesis) were simultaneously expressed in flax plants. The resulting transgenics had higher accumulation of phenolic acids and showed higher resistance to *Fusarium oxysporum* and *F. culmorum* (Lorenc-Kukuła et al. 2005). In a different approach, overexpression of glycosyltransferase was tried in flax. Enzymes of the glycosyltransferase family are required for the glycosylation of polyphenols which is the last step in their synthesis. The transgenic plants raised were found to show resistance to *Fusarium* infection and surprisingly the percentage of resistance was found to be much more than that of a case where flavonoid production was increased. It can thus be inferred that the higher presence of glycosyltransferase stabilized polyphenols which might be more effective than the simple overproduction of flavonoid compounds (Lorenc-Kukuła et al. 2009).

Transcription factors are proteins that regulate all aspects of a plant life, defense against invaders included. The use of transcription factor for increasing resistance of crop plants is an interesting idea because in this case the cloning of a single gene can regulate more than one response. The *NAC* family of transcription factors in plants plays a variety of role, majorly regulating genes involved in biotic and abiotic

stress. Gene silencing of *HvNAC* increased the susceptibility of barley to *Blumeria graminis* showing that it is a positive regulator of resistance (Jensen et al. 2008). *TGA* transcription factors are known to interact with *NPR1* at protein level and modulate SAR-associated genes. The *TGA2* overexpression in *Arabidopsis* showed enhanced resistance to *Peronospora parasitica*, an idea that can be further followed in non-model systems also (Kim and Delaney 2002). The *ERF* family of transcription factors has been known to have roles in resistance against different pathogens in a variety of crops. The *ERF1* of *Arabidopsis* is an early ethylene-responsive gene and transgenic plants that overexpress *ERF1* were rendered resistant to *B. cinerea* (Berrocal-Lobo and Molina 2004). The same principle was used with sea island cotton (*Gossypium barbadense*) *ERF*. The overexpression of a cotton *ERF* transcription factor *ERF2* regulates the expression of ethylene-responsive genes involved in defense response and enhances resistance against *Fusarium* wilt (Zuo et al. 2007). The *WRKY* family is another important family of transcription factors that is known to play a crucial role in stress responses. This has led to the cloning of WRKY factors in plants to enhance resistance. An example of this is the overexpression of *WRKY71* in banana leading to an enhanced resistance against *F. oxysporum*, the causative agent of *Fusarium* wilt (Shekhawat and Ganapathi 2013).

One of the early responses to pathogen attack on the plant is the oxidative burst leading to the production of reactive oxygen species (ROS), the imperative of which is famous in plant immunity. H_2O_2 is once such ROS with role in the start and spread of hypersensitive cell death. Potato transgenic plants overexpressing a *GO* gene that generates H_2O_2 were shown to express elevated levels and H_2O_2 and hence highly resistant against *Verticillium* wilt and *Alternaria* blight caused by *Verticillium dahliae* and *Alternaria solani* (Wu et al. 1997).

The use of antagonistic fungi like *Trichoderma* as agents of biological control of plant diseases is an idea that has been toyed with by many and utilized in many cases. When it comes to genetic engineering, it requires the use of a single gene leading to the question of what defines the antifungal characteristic of *Trichoderma*. Consequently, cell wall-degrading enzymes like chitinases and glucanases were purified from *Trichoderma* and were found to be responsible for its antifungal quality by degrading the fungal cell wall. The *ThEn-42*, a gene coding for a potent endochitinase from *Trichoderma harzianum*, was overexpressed in tobacco and potato plants surprisingly leading to an almost complete resistance in these two plants against *A. solani, A. alternata, B. cinerea*, and *R. solani* (Lorito et al. 1998). The *Venturia inaequalis* is a causative agent of apple scab, a disease that is responsible in a high measure for the extensive spraying of fungicides in commercial varieties of apples. The economic devastation of the disease is made worse by the development of fungal resistance against various fungicides. The "McIntosh" is a commercially available variety of apple that is susceptible to apple scab. An endochitinase gene (*ech42*) from *T. harzianum* was expressed in this variety and the new variety enhanced resistance against the apple scab causing fungus (Bolar et al. 2000). Overexpression of an endochitinase gene *CHIT42* from another fungus *Metarhizium anisopliae* in tobacco led to the plant's enhanced resistance against *Rhizoctonia solani* (Kern et al. 2010).

Fungi and oomycetes together form a group of pathogens which caused some of the most devastating diseases of crop plants. The diversity of this group has induced the need for diverse methods for engineering resistance. The emergence of resistant pathotypes has further hampered crop production seriously. The advancement in molecular biology has provided knowledge about the different genes and pathways that plants employ in order to resist against fungal pathogens and their subsequent use to produce transgenic crops. From *R* genes to transcription factors and from secondary metabolites to polygalacturonidases, the list goes on. The successful use of these genes in laboratory conditions provides hope for their future application in agriculture.

8.5 Nematodes

Plant parasitic nematodes can be at a very basic level divided into two classes – ectoparasites that live outside the host and the endoparasites that live and move inside the plant roots causing serious damage. The endoparasites of the group Heteroderidae are of importance considering the massive crop loss that they bring about. They can again be divided into two: the cyst nematodes and the root knot nematodes (Williamson 1999). The control of nematodes is dependent on a large part on nematicides; the environmental damage caused by which is monumental. Other conventional methods like crop rotation are impractical since they increase the area that needs to be brought under cultivation. Genetic engineering thus seems to be a possible solution for controlling plant parasitic nematodes.

The convenient discovery of *R* genes against the attack of parasitic nematodes has paved the way to their practical use in genetic engineering. One of the first *R* genes against nematodes was discovered in a wild variety of sugar beet *Beta procumbens*, shown to provide resistance against the cyst knot nematode *Heterodera schachtii*. This gene termed $HS1^{pro\text{-}1}$ renders the invading nematodes unable to complete their life cycles. Transfer of this gene from the resistant wild variety to a susceptible line showed enhanced resistance to the cyst nematode (Cai et al. 1997). The root knot nematode is another endoparasitic nematode that is known for its lethality as a parasite. A locus *Mi* from the wild variety of tomato *Lycopersicum peruvianum* provides resistance to the *Meloidogyne* spp. of root knot nematodes (Roberts et al. 1986). Sequencing of this locus identified a gene *Mi-1.2* which codes for a protein with an LRR region and a transmembrane domain (Milligan et al. 1998). Conveniently this gene provides for resistance not only against nematodes but also against aphids (Rossi et al. 1998) and whiteflies (Nombela et al. 2003). In order to check whether this gene could confer resistance in a genetic background different from tomato, *Mi-1.2* was transformed into eggplant (*Solanum melongena*). The resulting transgenic plants showed resistance not for aphids but the root knot nematode confirming the ability of *Mi-1.2* to provide resistance in other solanaceous

species (Goggin et al. 2006). *Hero* is another *R* gene of tomato that shows sequence similarity to *Mi-1.2*. A transgenic tomato line containing the *Hero* gene showed increased resistance to the cyst nematodes *Globodera rostochiensis* and *G. pallida* (Sobczak et al. 2005).

RNA silencing has already been discussed in this chapter as a potential tool for engineering resistance against viruses. The same has been tried against nematodes which is not surprising since the discovery that dsRNA corresponding to a DNA sequence can inhibit gene expression has been made in *Caenorhabditis elegans*, the famous model nematode. But parasitic nematodes start feeding only after they are established in the host plant, and hence the introduction of dsRNA before invasion is no cakewalk. Nonetheless, attempts have been made. The second stage of developing juveniles (J2) of two nematodes *Globodera pallida* and *Heterodera glycines* was made to take up dsRNA against various genes from a solution containing octopamine (a neuroactive compound that stimulates uptake). About 15 % of the nematodes ingested the solution as reported by the fluorescent marker Fluorescein isothiocyanate (FITC) (Urwin et al. 2002). Subsequently, two genes, calreticulin and polygalacturonase, which are involved in parasitism were targeted in the nematode *Meloidogyne incognita*. Uptake of dsRNA was induced by soaking in resorcinol (Rosso et al. 2005). The success of these attempts led to trials of actual transformation of plants to induce RNAi. Soybean roots were transformed with two RNAi constructs targeting a tyrosine phosphatase gene and a mitochondrial stress protein precursor. The formation of galls of *M. incognita* was reduced more than 90 % in the case of both the genes (Ibrahim et al. 2011).

Serine and cysteine protease inhibitors have been tried for engineering resistance against nematodes. A trypsin inhibitor from cowpea (*CpTI*) was transformed in potato and the transgenic plants were found to affect *Globodera pallida*. The infecting nematode population contained smaller and less harmful males (Hepher and Atkinson 1992). Field trial following the expression of chicken egg white cystatin in a susceptible potato resulted in a resistance of about 70 % (Urwin et al. 2001), whereas the expression of sunflower cystatin in the same cultivar led to lesser resistance against *G. pallida* (Urwin et al. 2003).

The agricultural use of transgenic nematode-resistant crops brings along with it all the concerns and controversies associated with GM crops. But here it has to be noted that the techniques employed are relatively much safer. Cloning of RNAi constructs pose less harm to the consumer and the environment since the genes targeted are specific for the development and establishment of nematodes. Likewise the use of protease inhibitors is also ideally safe. Egg white cystatin, for example, is consumed on an almost daily basis by humans who eat egg. This being said, the use of transgenic plants is an economic method for control of nematode pathogens especially in low-income economies where chemical methods and cultural methods prove impractical.

8.6 Insect Pests

When talking about genetically modified crops having resistance against insect pests, the first thing that comes into our mind irrespective of their profession or educational background is the Bt technology. *B. thuringinesis* (Bt) is a soil-dwelling bacterium, the hero of the tale. For years, agriculturists have known *B. thuringiensis* for its ability to produce a toxin that can kill insects. The use of the bacterium as biological agents of pest control has been applied since the early twentieth century. With advances in biotechnology, this aspect was broadened with the introduction of *cry* genes (the genes that are responsible for toxin production) into crops. The CRY toxin binds to specific receptors in the gut of the attacking insect and are solubilized leading to cell lysis and subsequent death (Daniel et al. 2001). The successful cloning of *Cry* genes was first reported in tomato and tobacco (Vaeck et al. 1987) following which the same technology has been employed in a variety of crop plants such as cotton, maize, papaya, and rice (Christou et al. 2006). Transgenic plants carrying the *cry* genes are the most commercially successful till date, but contrary to the common misconception, they are not the only ones tried. A lectin from garlic was expressed in tobacco plants which were tested for the efficiency of resistance against the aphid *Myzus persicae*. The survival of the aphid was found to be significantly reduced (Dutta et al. 2005). The cloning of a protease inhibitor in tobacco and subsequent greenhouse trials showed an enhanced resistance against *Frankliniella occidentalis*, commonly known as western flower thrip (Outchkourov et al. 2004). Likewise, the cloning of a barley trypsin inhibitor into the *indica* and *japonica* varieties of rice conferred resistance against the rice weevil *Sitophilus oryzae* (Alfonso-rubi et al. 2003). Recently, a group has demonstrated a promising strategy to counter the sap-sucking insects of order *Hemiptera* against which Bt toxins are not typically effective. This *Hadronyche versuta* derived neurotoxic peptide is insect-specific and act only within the hemocoel of insects. The group delivered it into the insect hemocoel by making chimeric protein of it with pea enation mosaic virus coat protein (Bonning et al. 2014). The various attempts and success of genetic engineering for resistance to insects suggest that despite the various controversies, it is a technology that has the potential to successfully control insect pests of plants.

8.7 Conclusion and Future Prospects

The genes and strategies that are used for engineering resistance are varied, but some of these seem to be common in different pathogens and pests (Fig. 8.1). The use of *R* genes, for example, is common to pathogens ranging from bacteria to nematodes. The same can be said for protease inhibitors. RNA interference is even more interesting and new avenues are being cleared in this area with the advancement in technology. The use of pathogen-derived resistance against viruses has

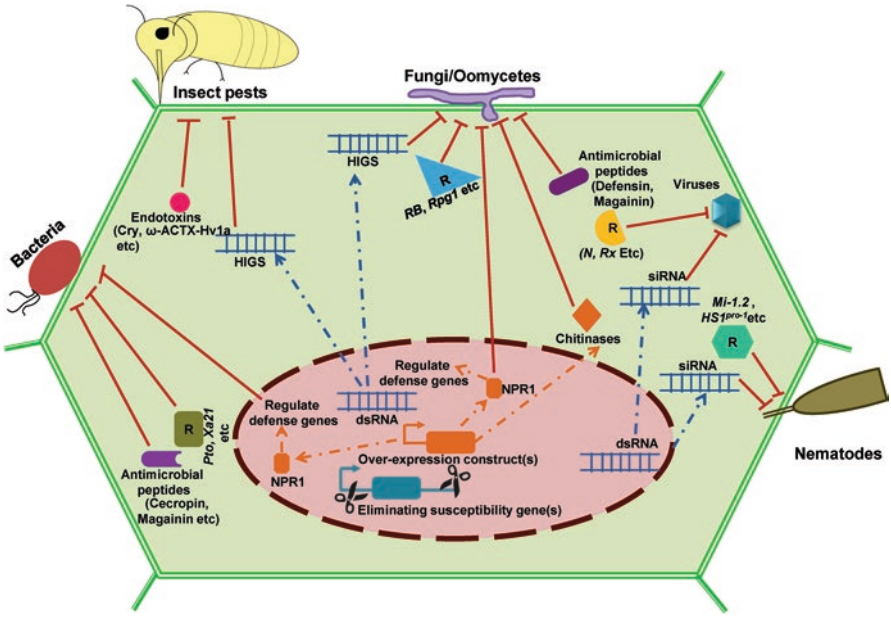

Fig. 8.1 Examples of the strategies employed for resistance against agents of biotic stress. The common strategies of genetic engineering used against various agents of biotic stress are highlighted. Some examples of genes/proteins involved in each strategy have been mentioned; *NPR1* Non-expressor of PR1, *HIGS* Host Induced Gene Silencing

brought about the idea of using the same technology against other pathogens. A recent strategy to be introduced is the HIGS (host-induced gene silencing), where a plant is transformed to express RNA silencing constructs that target genes of the invading pathogens (Nunes and Dean 2012). The technology has been successfully used against fungal pathogens such as *B. graminis* and *Fusarium verticillioides*. The specificity and efficiency of this technology seems to be promising, and it can very well be considered for different pathogens in the future.

Strategies used for improving resistance have always faced the same recurring problem: the development of resistance by the pathogens and pests. Conventional breeding programs have failed in many cases. The Ug99 strain of the wheat stem rust pathogen *Puccinia graminis f. sp. tritici* is one devastating example; their ability to overcome the *Sr* resistance genes have resulted in an uncontrollable spread of this pathogen across various areas under wheat cultivation (Singh et al. 2011). The same problem may also arise in case of transgenic plants. One advantage of genetic engineering over conventional breeding is that specific genes can be used. Further, multiple genes can be introduced in limited time in the same plant for enhanced resistance, especially against pathogens of different kind.

Another great concern about the use of GM crops is safety. The use of the term "genetically modified crops" seems to send a shiver down the spine of the general public across the world these days. Of all the technologies that humans have encoun-

tered, genetic engineering has received the most serious negative publicity, which is completely unaccounted for. Thus, most efforts in this area have been confined to the laboratories without ever seeing sunlight. To overcome this stagnation, problems that affect the consumer as well as the farmer need to be addressed. Millions across the world need to be convinced about the social, and not the industrial benefit of this technology. Thus, baby steps could be taken toward the elimination of problems faced in food productivity in the future.

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Chapter 9
Developing Stress-Tolerant Plants by Manipulating Components Involved in Oxidative Stress

Shweta Sharma, Usha Kiran, and Sudhir Kumar Sopory

Abstract Oxidative stress is one of the crucial outcomes of biotic and abiotic stress which leads to the physiological and metabolic alterations in plant system and, therefore, requires a balanced control of reactive oxygen species (ROS) production and its scavenging through antioxidative enzymes and proteins. The enzymatic components of antioxidative defense system consist of several enzymes including catalase (CAT), superoxide dismutase (SOD), and guaiacol peroxidase (GPX) and also the enzymes of ascorbate-glutathione (AsA-GSH) cycle, i.e., ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR). The nonenzymatic components include ascorbate (AsA) and glutathione (GSH) along with carotenoids and tocopherols along with other phenolic compounds. Exploiting the antioxidative behavior of these enzymes, several plants have been modified using transgenic techniques overexpressing the components of antioxidative stress pathways. Moreover, there are several other redox proteins, which have been genetically engineered to help plant survive in adverse conditions. This suggests that the development of transgenic plants overexpressing enzymes and redox-sensitive proteins associated with oxidative stress and antioxidative stress pathways will surely provide an important link of their role in tolerance to oxidative damage in crops. This chapter elucidates the recent advances in the defense system of plants during oxidative stress and also discusses the potential strategies for enhancing tolerance to oxidative stress.

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9.1 Introduction

Plants constantly face adverse environmental conditions that threaten their survival, and, thus, environmental stresses represent one of the most limiting factors for agricultural productivity worldwide. Abiotic stresses such as drought, salinity, flooding, heat, and cold disrupt the metabolic balance of cells, resulting into oxidative stress. Earliest signals in many abiotic stresses involve the accumulation of reactive oxygen species (ROS), which modify enzyme activity and gene regulation (Mittler et al. 2004). Other than this, the evolution of aerobic metabolic processes such as photosynthesis and respiration also unavoidably leads to the production of ROS in chloroplasts, mitochondria, and peroxisomes (Foyer 1997; Alscher et al. 1997). ROS are active both biochemically and biologically. They are responsible for destroying the cell membranes, nuclei, and cytoplasm (Brooker 2011). ROS includes a number of reactive molecules and free radicals derived from molecular oxygen where they act as a natural by-product of the normal metabolism of oxygen (Fridovich et al. 1995) and have important roles in cell signaling and homeostasis (Jones 2006; Foyer and Noctor 2005a, b, 2009; Hensley et al. 2000). During times of environmental stress (e.g., UV or heat exposure), however, ROS levels can increase dramatically (Devasagayam et al. 2004). Thus, oxidative stress is caused by an imbalance between the production and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage and accumulation of reactive oxygen species (ROS).

9.2 Reactive Oxygen Species: An Unavoidable Generation in Plants

In plants, oxidative stress is one of the major causes of damage in the system, as ROS are produced in both unstressed and stressed cells. However, the defense system, when presented with increased ROS formation under stress conditions, could be compromised. Another way of generation of ROS in plants is the activation of various oxidases and peroxidases under adverse conditions. ROS are predominantly produced in the chloroplast, mitochondria, and peroxisomes (Foyer et al. 1994). These organelles, however, have a strict balance between the production and scavenging of the ROS and maintain the equilibrium.

Chloroplast is one of the major sources of ROS production in plants (Asada. 2006). During photosynthesis, oxygen is continuously produced via light-driven electron transport and simultaneously removed from the chloroplast by assimilation (Asada 2006). There are two pathways responsible for ROS production in chloroplast. The first is the direct photoreduction of O2 to superoxide by reduced electron transport components associated with PSI, and the second pathway involves reactions taking place in photorespiratory cycle involving Rubisco in the chloroplast (Foyer and Noctor 2003).

Though the chloroplast is the major source of ROS production in the plant cell, the mitochondria contributes a major percentage of ROS burst in mammalian cells (Halliwell and Gutteridge 1999). The relative contribution of mitochondria in ROS production, however, is very low in case of plants (Rhoads et al. 2006). The major reason could be the presence of alternative oxidase (AOX) in mitochondria of plants that competes with the cytochrome complex for electrons and may reduce the production of ROS (Wagner and Krab 1995). In addition to the chloroplast and mitochondria, smooth endoplasmic reticulum, microsomes, and plasma membranes are involved in oxidative processes (Urban et al. 1997). They, however, produce ROS at lower levels. Further, peroxisomes and glyoxisomes compartmentalize enzymes involved in β-oxidation of fatty acids and C2-photorespiratory cycle, where glycolate transferase transfers electron to glycolate from oxygen and produces H_2O_2 ((Baker and Graham 2002; del Rio et al. 2002; Foyer and Noctor 2003).

9.3 Conditions Enhancing Oxidative Stress

Plants exposed to severe stress are more prone to photoinhibition leading to chlorosis. There are several herbicides which have been found to generate active oxygen species either by direct involvement in ROS production or by inhibiting biosynthetic pathways. One of the most important herbicides used these days is 2,4-dichlorophenoxyacetic acid (2,4-D). It is widely used as a weed control agent in agriculture, forestry, and lawn care practices. Romero-Puertas et al. (2004) have recently suggested that the herbicidal activity of 2,4-D may also be due to an increase in the production of ROS leading to oxidative stress in the weed. In addition to herbicides, the metals like Zn, Fe, Cr, Cu, and Cd also contribute to the ROS production in plants (Singh-Gill and Tuteja 2010). Other environmental factors which play major roles in the production of ROS in the system are drought, salinity, thermal stress, and light intensity (Sharma et al. 2012).

9.4 ROS Detoxification and Defense Against Oxidative Stress

In plant cells, specific ROS-producing and ROS-scavenging systems are found in different organelles such as the chloroplasts, mitochondria, and peroxisomes. These systems are well coordinated (Sharma et al. 2012). Under normal circumstances, ROS are generated at low levels. An appropriate balance between production and quenching of ROS is perturbed by a number of adverse environmental factors, giving rise to rapid increase in intracellular ROS (Noctor et al. 2002; Sharma et al. 2010), which, in turn, induces oxidative damage to proteins, lipids, and nucleic acids. In order to avoid the oxidative damage, there is an increase in the level of endogenous antioxidant defense comprising of nonenzymatic and enzymatic components to scavenge ROS (Sharma et al. 2010) (Fig. 9.1).

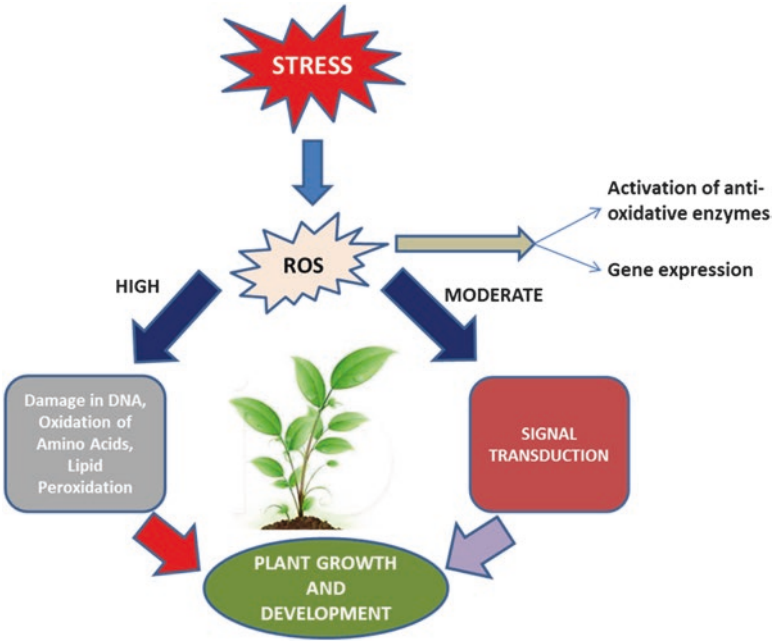

Fig. 9.1 Role of reactive oxygen species in plant cell signaling

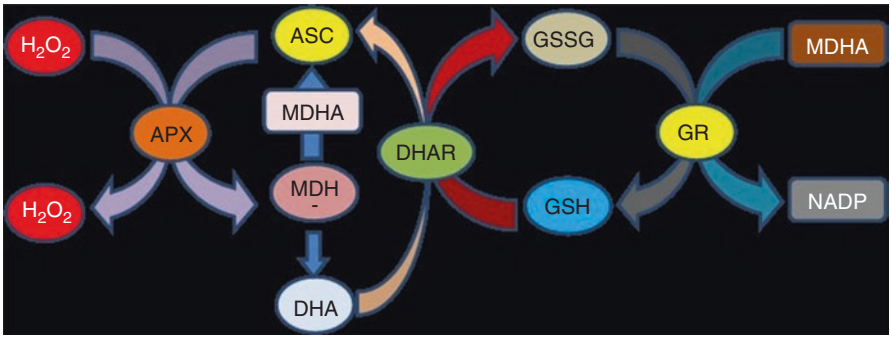

Fig. 9.2 The redox cycling of ascorbate in the chloroplast (Halliwell-Asada pathway)

The enzymatic components of the antioxidative defense system are comprised of several enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutathione (AsA-GSH) cycle, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Fig. 9.2). SODs are the first line of defense against oxidative stress. One of the first reactions against ROS production is its dissociation into oxygen and H_2O_2 (Bowler et al. 1991). However, this

reaction only converts one ROS to another, i.e., H_2O_2, which also needs to be destroyed as it promptly attacks thiol proteins (Noctor and Foyer 1998).

Plants also contain several types of H_2O_2 degrading enzymes, of which catalases are unique as they do not require cellular reducing equivalent. Other than catalases, ascorbate-glutathione (AsA-GSH) cycle, also referred as Halliwell-Asada pathway, is the recycling pathway of ascorbate and glutathione regeneration and detoxifies H_2O_2 (Tayefi-Nasrabadi et al. 2011). AsA-GSH cycle plays an important role in combating oxidative stress induced by environmental stresses (Sharma et al. 2012).

Nonenzymatic components of the antioxidative defense system include the major cellular redox buffers such as ascorbate (AsA) and glutathione (γ-glutamyl-cysteinyl-glycine, GSH) along with tocopherol, carotenoids, and phenolic compounds. These interact with numerous cellular components and play crucial roles in defense and also as enzyme cofactors. The antioxidants influence plant growth and development by modulating processes from mitosis and cell elongation to senescence and cell death (Pinto et al. 2004). Mutants with decreased nonenzymatic antioxidant contents have been shown to be hypersensitive to stress (Semchuk et al. 2009).

It has been shown in earlier studies that proline, a proteinogenic amino acid, modulates enzymatic antioxidant activities and nonenzymatic antioxidant contents in plants to protect them from oxidative stress induced by abiotic and biotic stresses. This function of proline has been attributed to its ROS scavenging and singlet oxygen quenching activities (Smirnoff and Cumbes 1989; Matysik et al. 2002; Iqbal and Ashraf 2007). In addition to direct ROS scavenging, proline was also found to modulate the ROS and MG detoxification pathways, inducing oxidative stress tolerance. The synthesis of proline generates NADP, whereas NADPH is produced during proline oxidation. Therefore, the proline biosynthesis and degradation cycle is crucial for buffering redox potential in different cellular organelles, such as the cytosol, mitochondria, and plastids. Abiotic stress-induced accumulation of proline has also been found to influence the synthesis of the multifunctional antioxidant glutathione (GSH), probably because the two molecules have a common precursor, glutamate (Kocsy et al. 2005). Interestingly, transgenic plants overexpressing osmotin showed tolerance to abiotic stresses accompanied with higher levels of proline even when they were not exposed to stress (Husaini and Abdin 2008).

9.5 ROS in Plant Cell Signaling

Though ROS were initially recognized as the toxic by-products of aerobic metabolism, it has now become apparent that they are an important component of cell suicide, antimicrobial compound, lignification of substrate, and, most importantly, cell signaling by acting as cell signal transducer (Mehdy 1994). ROS-mediated signaling is strongly controlled by a tight balance between the production of ROS and its

scavenging, simultaneously. The production of ROS in the chloroplast and mitochondria induces the changes in the nuclear transcriptome demonstrating that the information is transmitted from these organelles to the nucleus. Out of all the ROS, H_2O_2 is shown to be the most active signaling molecule, and its accumulation leads to a variety of cellular responses (Kovalchuk 2010; Bhattacharjee 2005; Halliwell 2006; del Rio et al. 2006). The signaling role of H_2O_2 is also reported in response to various stresses. In winter wheat, low concentration of H_2O_2 induces the synthesis of small polypeptides which protects the cell from chilling stress (Matsuda et al. 1994). Similar results have also been proposed in maize, where a pretreatment of maize seedlings with low concentration of H_2O_2 leads to the chilling tolerance of plant (Prasad et al. 1995). ROS also play a major role in the activation of a large number of signal-transducing molecules under stress such as ethylene, ABA, and salicylic acid. They have also been involved in the photooxidative stress, where its higher concentration activates the degradation of proteins involved in light-harvesting complex II (LHCII). This, in turn, causes the photoinhibition of photosynthetic electron flow and, thus, less ROS in the system (Bhattacharjee 2012). ROS are also shown to be an important factor in plant-pathogen interaction (Levine et al. 1994). In *Arabidopsis*, expression of defense-related genes encoding phenylalanine ammonia lyase (PAL) and glutathione S-transferase (GST) has been reported to be expressed in response to oxidative stress (Lopez-Delgado et al. 1998). The induction of lipid peroxidation, cell metabolism, and growth and morphogenesis of plant cells are other significant roles of ROS (Fig. 9.3). Therefore, ROS display protection against stress by activating the antioxidative mechanism either by modulating the gene expression or activities of the enzymatic proteins (Desikan et al. 1999; Desikan et al. 2000). Altogether, the oxidative burst in the plant can trigger protective pathways against environmental stresses and, thus, play an important role in plant defense (Bhattacharjee 2012).

9.6 Manipulation and Development of Transgenic Plants Overexpressing Genes Involved in Oxidative Stress Defense System

To reduce oxidative damage in plants, manipulation of ROS-scavenging enzymes has been done by using gene transfer technology for which several molecular approaches have been exploited (McKersie et al. 1993, 2000; Sen Gupta et al. 1993; Allen et al. 1997; Payton et al. 1997; Chen et al. 2003). Recently, overexpression of ACO genes of tomato has been found to induce signaling pathway (Lin et al. 2011). As discussed above, there are three major enzymes involved in the protection pathway from oxidative stress in plants, viz., superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR).

Superoxide dismutases catalyze the dismutation of superoxide (O_2-) into oxygen and hydrogen peroxide. In the early 1990s, transgenic tobacco plants overex-

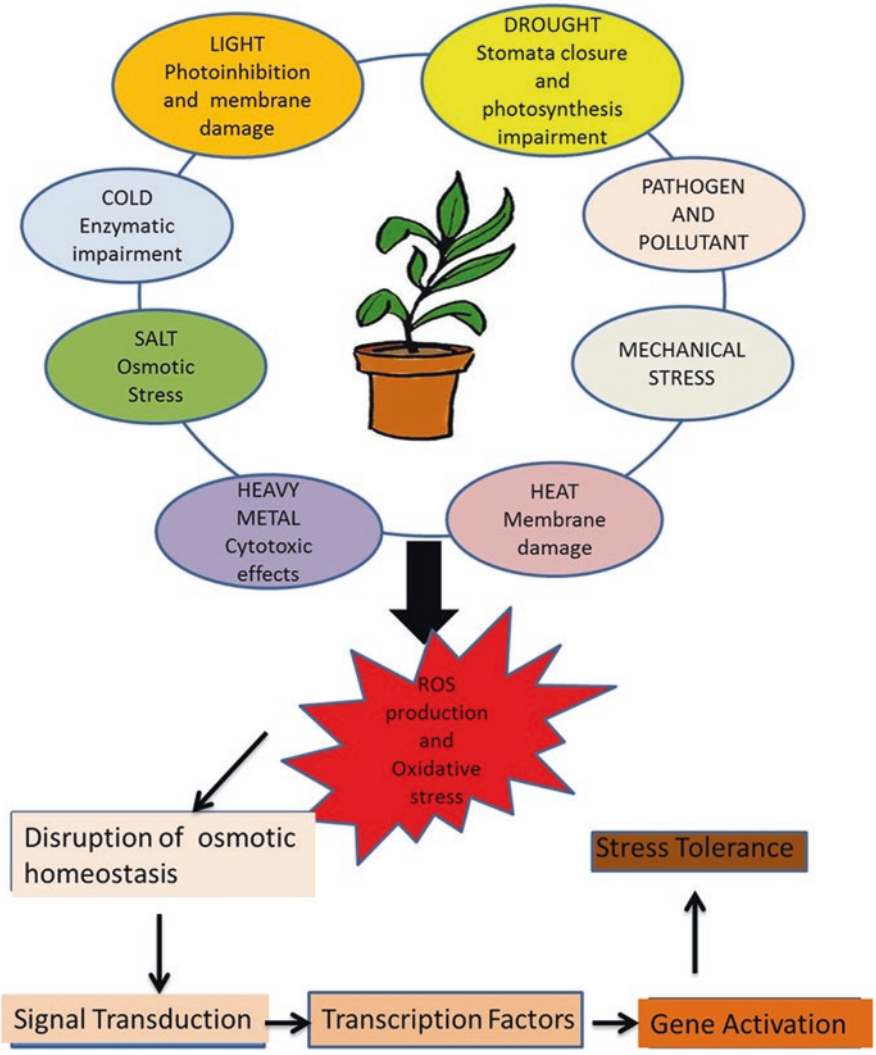

Fig. 9.3 Environmental stress, ROS burst, and defense mechanism adapted by plants

pressing chloroplast-localized Cu/ZnSOD from petunia were developed, which showed higher SOD activity than untransformed control plants. In a similar experiment, increased SOD activity was observed in tobacco (*Nicotiana tabacum*) overexpressing (*Pisum sativum*) chloroplastic Cu/ZnSOD resulting in significant increase in resistance to light-mediated methyl viologen (MV)-induced membrane damage (Tepperman and Dunsmuir 1990). It has also been reported that overproduction of FeSOD in the chloroplasts of tobacco protects both the plasmalemma and PSII from MV-induced damage (VanCamp et al. 1996). Also, the transgenic tobacco overproducing SOD in the chloroplasts exhibits an enhanced tolerance to

chilling in the dark (Foyer et al. 1994) or in the light (Sen Gupta et al. 1993). An enhanced tolerance to freezing stress in transgenic alfalfa overproducing SOD in the chloroplasts has also been reported (McKersie et al. 1993). Transgenic potato overexpressing Cu/ZnSOD enhances the oxidative stress defense (Perl et al. 1993). It has also been observed that elevated levels of superoxide dismutase protect transgenic plants against ozone damage in *Nicotiana plumbaginifolia* (Van Camp et al. 1994). Transgenic tobacco plants that overexpressed pea Cu/ZnSOD II have been shown to compensate for the increased levels of SOD with increased expression of the H_2O_2-scavenging enzyme APX (Gupta et al. 1993).

Transgenic tobacco plants overexpressing manganese superoxide dismutase (MnSOD) have been reported to have significantly reduced amount of cellular damage (Bowler et al. 1991). Overexpression of the chloroplastic MnSOD in alfalfa (*Medicago sativa*) and cotton (*Gossypium hirsutum*) showed increased chilling tolerance (McKersie et al. 1993; Trolinder and Allen 1994). Sugar beets carrying superoxide dismutase transgenes have been reported to exhibit increased tolerance to methyl viologen (Konstantinos et al. 2010). It is also shown that rice transformed with yeast MnSOD was more tolerant to salt stress as compared to the non-transformed plants (Tanaka et al. 1982). Later, it was reported that Cu/ZnSOD from rice can facilitate salt stress management in tobacco (Badawi et al. 2004). Recently, overexpression of cytosolic Cu/ZnSOD from a mangrove plant has been shown to confer abiotic stress tolerance in rice (Prashanth et al. 2008).

APXs have high substrate specificity for ascorbate and are the primary H_2O_2-scavenging enzymes in the chloroplasts and cytosol of plant cells (Asada 1992). Transgenic tobacco plants overexpressing cytosolic APX have been developed (Pitcher et al. 1994; Sen Gupta et al. 1993), which showed enhanced defense against oxidative stress as compared to the non-transformed plants. Overexpression of APX gene from *Arabidopsis* in tobacco has shown to confer protection against oxidative stress in transgenic plants (Wang et al. 1999). Glutathione reductase is another important detox enzyme. Transgenic tobacco plants that overexpressed GR showed higher tolerance for MV and were able to maintain the reduction state of their ascorbate pools more effectively than control plants (Foyer et al. 1991; Aono et al. 1993). Similarly, transgenic tobacco plants overexpressing pea GR have also been found to contribute against oxidative stress better than untransformed plants (Creissen et al. 1994).

Hyperaccumulation of proline by P5CS overexpression in transgenic algae and tobacco plants were shown to reduce free radical levels suggesting ROS-scavenging activity of proline. The damaging effects of singlet oxygen and hydroxyl radicals on photosystem II (PSII) were reduced by proline in isolated thylakoid membranes (PSII) (Alia and Mohanty 1997). Proline treatment diminished ROS levels in fungi and yeast preventing the programmed cell death and reduced lipid peroxidation in alga cells exposed to heavy metals (Chen and Dickman 2005; Mehta and Gaur 1999). Additionally P5CS1 mutants showed strongly reduced proline accumulation in response to stress, concomitantly with reduced root growth, enhanced production of ROS in leaves, and altered NADPH ratio (Szekely et al. 2008; Sharma et al. 2012). Transgenic plants with higher proline synthesis display improved tolerance

to various abiotic stresses including oxidative damage. Recently, exogenous application of proline (10 mM) to seedlings of Thai aromatic rice (cv. KDML105; salt sensitive) during salt stress and subsequent recovery showed upregulation of genes encoding the antioxidant enzymes Cu/ZnSOD, MnSOD, CytAPX, and CatC (Nounjan and Theerakulpisut 2012). Importantly, an upregulation of proline synthesis genes (P5CS and P5CR) in response to exogenous proline application was also seen. By contrast, compromised proline accumulation in P5CS1 insertion mutants led to accumulation of ROS and enhanced oxidative damage (Szekely et al. 2008). Being an osmolyte, alternatively, proline can protect and stabilize ROS-scavenging enzymes and activate alternative detoxification pathways. In salt-stressed tobacco cells, proline increased the activities of methylglyoxal detoxification enzymes; enhanced peroxidase, glutathione S-transferase, superoxide dismutase, and catalase activities; and increased the glutathione redox state (Hoque et al. (2008), Islam et al. (2009)). In the desert plant *Pancratium maritimum*, catalase and peroxidase were found to be stabilized by proline during salt stress (Khedr et al. (2003)). The salt-hypersensitive P5CS1 *Arabidopsis* mutant shows reduced activities of key antioxidant enzymes of the glutathione-ascorbate cycle, leading to hyperaccumulation of H_2O_2, enhanced lipid peroxidation, and chlorophyll damage (Szekely et al. 2008).

9.7 Gene Pyramiding for Oxidative Stress Tolerance

Despite the development of several transgenic plants overexpressing components of oxidative stress defense, the protective effects provided by the overexpression of single genes are usually rather small. Thus, there is a scope of gene pyramiding in the plants and observing the effects of combining the genes in tolerance against oxidative stress. The transgenic tobacco plants overexpressing Cu/ZnSOD and APX showed elevated tolerance toward oxidative stress, induced by MV (Kwon et al. 2002). Same group also reported that the transgenic plants expressing human DHAR in their chloroplasts had high AsA levels and showed increased tolerance to both MV-induced oxidative stress and salt stress (Kwon et al. 2003). Transgenic potatoes and sweet potatoes, expressing chloroplast Cu/ZnSOD and APX, have also been developed. The expression of these antioxidant enzymes conferred increased protection against MV-induced damage and salt-induced damage, indicating that manipulation of antioxidant mechanisms in chloroplasts is important in the protection of plant cells against multiple environmental stresses (Tang et al. 2006; Lim et al. 2004).

Overexpression of radical-scavenging enzymes such as SOD and GR in plants has also been shown to make them resistant to drought, ozone, and low-temperature and high-light stresses (Gupta et al. 1993; Van Camp et al. 1994; McKersie et al. 1996). According to a recent report, co-expression of GST and CAT positively affects the AsA-GSH cycle and coordinates upregulation of AsA-GSH pathway enzymes rendering the plants more tolerant to Cd and heat-induced oxidative stress (Zhou et al. 2008). Additionally, tobacco plants overexpressing three antioxidant

enzymes, Cu/ZnSOD, APX, and DHAR, showed greater tolerance to oxidative stress than double transgenic plants overexpressing Cu/ZnSOD and APX (Lee et al. 2007). The tolerance mechanism against oxidative stress induced by various abiotic stresses has been investigated in transgenic potato tubers overexpressing D-galacturonic acid reductase (*GalUR*) gene. The overexpression of this gene in potato results into an enhanced accumulation of AsA and thus helping the plants to tolerate oxidative stress (Hemavathi et al. 2010). Enhanced tolerance of transgenic potato plants expressing both SOD and APX has also been reported in response to oxidative stress (Tang et al. 2006).

Thus, it can be postulated that development of transgenic plants that overexpress enzymes involved in oxidative stress and damage provides an insight into the role clue of enhancing the ROS-scavenging systems of crop plants.

9.8 Novel Proteins Involved in the Protection of Plants from Oxidative Burst

The other important ROS-triggered defense system comprises the activation of some specific genes leading to specific acclamatory responses. One such example is the boosting of antioxidative defense system by stimulating the expression of antioxidative enzymes and by inducing the stress responsive proteins in *Arabidopsis* (Santos et al. 1996; Karpinski et al. 1997). In response to stress, plants sense, transduce, and translate specific proteins called redox-sensitive proteins which are operated by reversible oxidation/reduction maintaining the cellular redox state. Glutathione or thioredoxins are the best studied redox-sensitive molecules which control the redox state of the cell (Rouhier et al. 2008; Mylona and Polidoros 2010). Redox-sensitive proteins generally perform their functions by activating other signaling components such as transcription factors, kinases, and phosphatases. Other than this, plants also possess chloroplastic, mitochondrial, and cytosolic redox-regulating system to control gene expression (Mahalingam and Fedoroff 2003; Nishiyama et al. 2001).

There are several redox-sensitive transcriptional factors also whose activities are relied on redox- and structure-dependent manner (Tron et al. 2002; Heine et al. 2004; Serpa et al. 2007; Shaikhali et al. 2008, 2012). The best example is the redox-dependent transcriptional factor, Rap2.4a which, under oxidizing conditions, switches its structure from monomer and dimer to polymers, respectively, where the dimeric form of Rap2.4a is found to play an important role as a transcription factor to stimulate nuclear gene expression of the photosynthetic chloroplast enzymes (Shaikhali et al. 2008). Another example is basic leucine zipper (bZIP) transcription factor in *Arabidopsis*, AtbZIP16, which contains a conserved Cys residues that play a critical role in redox regulation of the target gene expression, which has been shown in transgenic *Arabidopsis* overexpressing the Cys-mutated variant of bZIP16 (Shaikhali et al. 2012).

There are other important proteins which act by interacting with several transcription factors and defending the plants from oxidative stress. One such example is radical-induced cell death (RCD1) protein which performs its function by interacting with various transcriptional factors and proteins as well. RCD1 has also been established as an important regulator of stress and hormonal and developmental responses in *Arabidopsis thaliana* (Overmyer et al. 2005). RCD1 was also found to interact with several transcription factors in order to protect the plants from stress (Castillon et al. 2005; Indorf et al. 2007). Recently, we have also shown that RCD1 interactas with RNA binding domain containing protein (OsRBD) and confers enhanced stress tolerance in yeast and may play an important role under abiotic stress responses in plants (Sharma et al. 2016).

9.9 Conclusion and Future Perspective

Plant cells produce various kinds of reactive oxygen species (ROS) during the course of normal aerobic metabolism or when an organism is exposed to a variety of stress which causes a widespread damage to intracellular macromolecules leading to the degenerative diseases. To cope with oxidative stress, plants generate redox gradient and elicit the inactivation of the oxidative burst-generating enzymes (Pignocchi and Foyer 2003; Suzuki et al. 2012). The redox-dependent functional switching is a typical scheme for the plant defense systems. The transgenic plants overexpressing genes involved in oxidative stress defense mechanism help plants to survive in adverse conditions. Thus, the valuable insights into plant responses induced by biotic/abiotic stresses in terms of the rapid change in redox potential at the molecular level would help scientists to enhance plant performance, yield, and productivity, even under adverse conditions.

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Chapter 10
Plant Adaptation in Mountain Ecosystem

Sanjay Kumar and Surender Kumar Vats

Abstract Plant adaptation studies in mountains are important since the environmental conditions change with altitude and are often termed 'harsh' at high altitude (HA). This calls for a degree of specialization at structural and functional levels in plants inhabiting these locations. Altitudinal gradients thus illustrate the ability as well as limitation of plant species to adapt to environmental changes. HA environment also draws distinction for being most responsive to climate change, though the predicted trends remain the most complex and uncertain. The high climatic and microclimatic variability reported within different mountain ecosystems of the world further adds to this complexity. The issue of plant adaptation to the changing environmental variables with altitude has evoked high research interest, particularly in the context of changing climatic pattern across the globe. Understanding biological responses of HA species to extreme and fluctuating environmental conditions is fundamental to explain species adaptive potential and capacities, in order to predict their future redistribution (e.g. up-migration), changes in phenology and life cycle pattern and changes in secondary metabolites, to say a few. The present chapter draws attention to the basis of plant adaptation, with reference to plant species in western Himalaya, at physiological, morphological, molecular and biochemical (secondary metabolites) levels in context to change in altitude. These studies can predict the targets for further manipulation of genetic makeup of HA species under the climate change scenario in mountains.

10.1 Introduction

Plant adaptation studies in mountains are exciting due to large variations in environmental variables, across relatively short altitudinal distances. High altitude (HA) brings substantial changes in numerous environmental parameters such as higher irradiance levels; larger diurnal fluctuations of temperature; at times limited water and nutrient supply; higher velocity of wind; declining partial pressure, mainly of

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CO_2; and short time period for growth and development. Other factors that may influence mountain environment are aspect, topography and canopy cover which can modify specific habitats in terms of soil moisture content, relative humidity, available sunshine and wind velocity within the same elevation. Change in aspect influences available sunshine and precipitation and thus can considerably alter soil temperature and moisture. In the western Himalaya, rainfall was reported to be more than double for windward side of Beas basin in the western Himalaya compared to the leeward side, though overall trend was reported to decrease linearly with elevation for middle Himalayan range (Singh and Kumar 1997). Total precipitation can also be influenced by change in geographic conditions such as high geographic barriers that forbid monsoon from Indian plains which render vast expanse of trans-Himalaya into cold desert. These factors combine to influence environment and bring about extreme microclimatic variations to give selective performance advantage to some species. The creation of isolated habitats and conditions may promote development of new species/sub-species and explain the high degree of endemism reported in mountains.

Mountain environment has another distinction that large variations may occur in environmental variables across relatively a much smaller distance; such differences along latitude are located much far away. For instance, alpine and tropical ecosystems, representing the two sets of climatic 'extremes', are separated apart by merely a few (3–4) kilometres in the Himalaya, compared to a few hundred to thousand kilometres latitude-wise. Mountains thus provide a rare opportunity to study plant adaptive diversity, occurring naturally along the continuum of changing environmental variables over short spatial distance.

Plant species distributed in a particular environment exhibit their affinity to the prevailing set of environmental variables. The same species spread across heterogeneous conditions of changing environment must harbour adequate potential to adapt to the changing conditions across large continuum of variability. Plant species may do so by invoking phenotypic plasticity that often helps species to tackle environmental heterogeneity through changes in biochemical, physiological and molecular response at different life stage levels, primarily to optimize plant performance along the changing altitudinal gradient (Kumar et al. 2005, 2006a, b; Geng et al. 2007; Gratani 2014; Merilä and Hendry 2014). At times, differences in environmental parameters can be large for the phenotypic variability to confer fitness, and such plant populations may develop genetic variation to adapt to the changing environments.

Overall, species response to survive and reproduce under extreme conditions is referred to as its "fitness", and this ability to fit in a specific environment can be achieved through different strategies: (1) by adaption following evolutionary (phylogenetic) adjustments, (2) by nonreversible modifications of life cycle events of an individual that are not inherent and (3) by plants' capacity to acclimate, the response being reversible (Körner 2003). At times, phenotypic plasticity may not be sufficient to help species to extend their geographical distribution range and invoke genetic modifications to create new "ecotypes". The concept of ecotype has been used quite

early in mountain plant ecology (Billings 1957). It is generally believed that altitudinal ecotypes modify more specifically to suit to their native environment.

Ecotypes are known to be better adapted to conditions of their local environment, and such a behaviour has been observed for altitudinal populations of different species in mountains (Galen et al. 1991). The genetic differentiation therefore can be viewed as specialization to adapt best to conditions of local environmental (Van Tienderen and Van der Toorn 1991; Galen et al. 1991).

10.2 Altitude and the Changing Cues

The fact that tree vegetation gets restricted up to tree line in mountains indicates the growing environmental stress with altitude, with peak stress experienced by a species at its highest limit of distribution. HA sites are usually such locations and represents about one-fifth of the total terrestrial area of globe. Meanwhile, it may be pointed out that HA environment, which is termed as 'harsh', may not be stressful for plants dwelling naturally or adapted to these environments (Korner 2006). For instance, most alpine species may not necessarily be under the so-called stress (Korner 2006).

HA species with narrow altitudinal range of distribution flourish in their native environment, but fail to grow under 'less stressful' conditions of lower elevation. Some of the potential stress inducing environmental variables that change with altitude are partial pressure of gases (CO_2), temperature, irradiance, etc. Partial pressure of air drops with rise in altitude roughly at the rate of 1 millibar (0.1 kilopascal) per 10 m rise in elevation (Kumar et al. 2004). This drop in CO_2 levels has biological implications, such that it can substantially reduce photosynthetic productivity in C_3 plants, particularly at higher temperatures and during drought stress (Sage and Coleman 2001). The possibility that these low levels of CO_2 could suppress the net rate of photosynthesis (P_N) at HA has been expressed by several researchers over a period of time (Decker 1959; Billings et al. 1961; Mooney et al. 1966; Friend and Woodward 1990). Acclimation of photosynthesis at reduced CO_2 levels is likely to help plants to optimize their performance. Also, the drop in partial pressure causes air to expand. Expansion of air is an endothermic process that extracts heat from surroundings and leads to cooling. Temperature may occur at about 1 °C with a rise of every 270 m in altitude (Singh and Singh 1987). In Alps, the rate of drop in temperature range from 0.55 to 0.62 °C per 100 m rise in elevation during summer months (Larcher 2012). Temperature regime in mountain ecosystem may also change with aspect or other microclimatic factors. Temperature may cross well over 40 °C on a sun facing high-altitude slope during summer (Larcher et al. 2010), whereas within the same plant, as in the case of *Primula minima*, tissue temperature may change from 4 °C at sunrise to 34 °C during early afternoon (Larcher 2012). Therefore, the plant species dwelling therein need to withstand high as well as low temperature (Lütz et al. 2010). Other than air or shoot temperature, soil temperature in mountains has shown wide variations across different microsites, during different

months or seasons. The minimum and maximum temperature at a HA site could vary from 0 to 28 °C (Larcher 2012).

Spitaler et al. (2008) reported that values of total irradiance, ultraviolet (UV)-A and UV-B increased by 8 %, 9 % and 18 %, respectively, per 1000 m rise in elevation. UV-B radiation, which has the potential to cause damage to plants and animals, was shown to increase by approximately 10 % with rise in 1000 m elevation in Alps (Körner 1999). Similar values for the Himalaya are not known. Across the same altitudinal distance, biologically effective radiation was stated to increase by 15–20 % (Turunen and Latola 2005). Photosynthetic photon flux density (PPFD) studied across a range of 2000 m in Alps showed no substantial difference with change in elevation (Körner and Diemer 1987). Data recorded in the Himalaya at HA (Kumar et al. 2005) and by some other studies have shown higher values of PPFD at high elevation (Clements et al. 1950; McElwain 2004) and are best explained due to the difference in air turbidity at different altitudes. Irrespective of the altitude, total sunshine hours may differ considerably on the north and south faces of high Himalaya and due to persistence or absence of cloud cover. Similar differences are well documented for different mountain ecosystems of the world (Friend and Woodward 1990). Therefore, environmental variables across relatively small altitudinal scale may significantly influence species distribution and plant stature, structure, biochemistry, morphology and physiology (Larcher 1995; Purohit 2003).

10.3 Altitude and Adaptive Variability

HA plants display different strategies to cope with 'extreme' environmental conditions. These may be through invoking specialized physiological processes, which include efficient carbon assimilation, reduced photoinhibition, high capacity of reactive oxygen species (ROS) detoxification, altered chemical composition of plants, capacity to synthesize special lipids capable of modifying cell membranes for providing greater flexibility and water permeability, production of carbohydrates with antifreeze properties, filters to protect against UV radiation, antioxidants and quenchers for free radical scavenging which are not observed in case of low-altitude plants (Joshi 1987).

10.3.1 Response to Low Partial Pressure of CO_2

The ecological significance of low CO_2 in context to plants relates to the geological era of the Last Glacial Maximum (18,000–20,000 years ago) when atmospheric concentration of CO_2 dropped below 200 ppm (Tissue and Lewis 2012). Low CO_2 is known to adversely impact photosynthesis and plant productivity. At 180 ppm of

CO_2, photosynthetic capacity is reduced to nearly half of that at the current CO_2 levels (Sage 1995). Low CO_2 becomes limiting as a substrate for the carboxylation reaction of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) and also leads to greater photorespiratory losses. To overcome this limitation, plants need to acclimate and may undergo varied adaptive strategies, such as different carbon-concentrating mechanisms observed in aquatic and terrestrial plants (Vats et al. 2011).

Partial pressure of CO_2 drops with increase in elevation that may affect photosynthetic performance of plants at HA. Some clues in this regard can be seen in the altitude-related increase in activity of the Rubisco enzyme (Pandey et al. 1984) and the enhanced efficiency of carbon uptake (Körner and Diemer 1987; Körner and Diemer 1994; Kumar et al. 2005; Vats and Kumar 2006). Activation state of Rubisco provides important clue to determine net photosynthetic rate (P_N). At HA activation state of Rubisco was lower suggesting the role of low partial pressure of CO_2. Enhanced efficiency of CO_2 uptake as a function of increasing altitude was reported in wild species of *Rumex nepalensis* and *Trifolium repens* and in cultivars of *Hordeum vulgare* and *Triticum aestivum* when grown at HA (Kumar et al. 2005).

A shift in photosynthetic metabolism triggered at HA was reported by us in crop plants wheat and barley (Kumar et al. 2006a, b) and wild plant *Rumex nepalensis* (Kumar et al. 2008). To understand this shift, primary products of photosynthesis were analysed using radiotracer, and activities of enzymes of carbon and nitrogen metabolism were studied in barley and wheat at different altitudes. Our data showed significantly higher carboxylase and oxygenase activities of Rubisco, phosphoenolpyruvate carboxylase (PEPCase), aspartate aminotransferase (AspAT) and glutamine synthetase (GS) at high altitude as compared to those at low altitude (Kumar et al. 2006a, b). It was suggested for these C_3 plants that PEPCase sequestered CO_2 from atmosphere and/or that generated metabolically in parallel to Rubisco. The PEPCase-mediated production of oxaloacetate was suggested to be channelized for aspartate synthesis, using glutamate as a source of ammonia. This cycle operative in the HA environment has the potential to conserve carbon and nitrogen (Fig. 10.1) (Kumar et al. 2006a, b, 2008; Vats et al. 2011; Kaachra et al. 2011). The process of photorespiration/amino acid catabolism could be the source of ammonia. The mechanism (Fig. 10.1) while operates photorespiration does not eventually end up in loss of carbon and nitrogen. Any efficient photosynthetic uptake is a useful strategy to compensate for the shorter growing period at high altitudes, and retention of the same would have functional advantage under elevated CO_2 environment.

10.3.2 Response to Irradiance

Plants at HA locations mostly grow in open habitat conditions and are exposed to high irradiance levels Kumar et al. (2007). Some of the earlier studies in Alps showed that maximal photosynthetic rates in leaf of high-altitude plants were achieved at high PPFD (Tranquillini 1964; Billings and Mooney 1968). In native

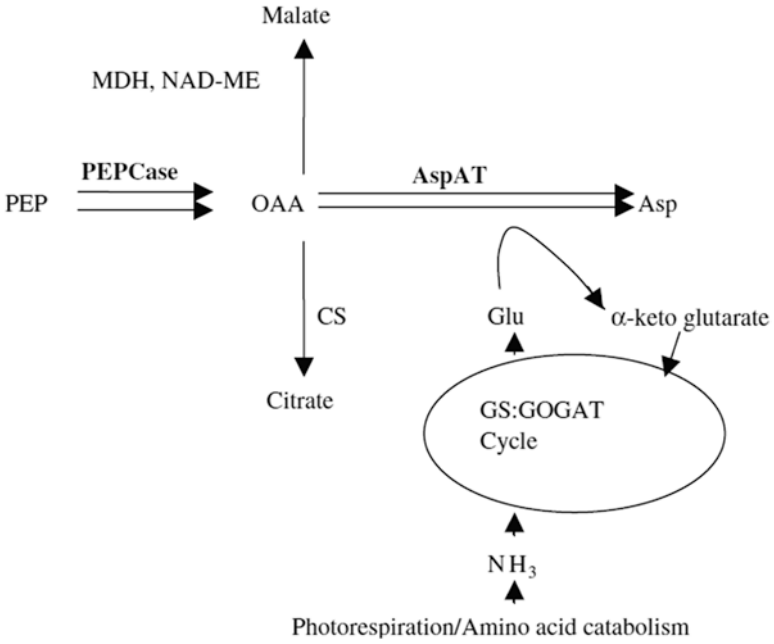

Fig.10.1 A mechanism to fix higher CO_2 at low partial pressure of CO_2. The mechanism has been reproduced based on the work at high altitude which is characterized by the environment of low partial pressure of CO_2 and other gases, with the net photosynthetic rates remaining comparable to the plants at low altitude (Reproduced from Kumar et al. 2005; permission granted by the publisher; licence number 3556301012088 dated 26th Jan, 2015). *Asp* aspartate, *AspAT* aspartate aminotransferase, *CS* citrate synthase, *Glu* glutamic acid, *GOGAT* glutamine:2-oxoglutarate aminotransferase, *GS* glutamine synthetase, *MDH* malate dehydrogenase, *NAD-ME* NAD-malic enzyme, *OAA* oxaloacetate, *PEP* phosphoenolpyruvate, *PEPCase* phosphoenolpyruvate carboxylase, *Rubisco* ribulose-1,5-bisphosphate carboxylase/oxygenase

population of *Mimulus cardinalis* studied at different elevations, it was reported that light-saturated rates of photosynthesis increased linearly with altitude (Milner and Hiesey 1964). HA plants are generally credited with better light use efficiency. Körner and Diemer (1987) reported that alpine plants achieved 50 % of photosynthetic capacity at one-tenth of full sunlight which is less than required by plants from the lowland.

There is evidence to show that alpine timberline plants are adapted to tolerate UV-B radiation stress (Turunen and Latola 2005). Compared to low-altitude plants, these plants exhibit greater leaf surface reflectance, epidermal thickness, UV-B-absorbing compounds in leaf surface tissue or organelles and better oxidative stress defence (Jordan 2002; Lavola 1998). Most morphological leaf structural (cuticle thickness, the amount of epicuticular waxes and wettability) characteristics are greatly influenced by change in microclimatic and edaphic factors and may decrease, increase or remain unchanged with increasing altitude.

On the contrary, plants at low altitude may experience lower levels of PPFDs due to greater turbidity in air that reduces transmissibility of light. HA locations however at times may also experience rapidly changing light and shade environment due to moving clouds. Given that HA plants need to be photosynthetically efficient (Streb et al. 1998) for having a shorter period available for active growth, frequent stomatal closure would greatly undermine the photosynthetic output. This has bearing on the light-regulated behaviour of stomata. Our study in HA population of *R. nepalensis* and *T. repens* in western Himalaya showed no significant change in stomatal conductance with changing PPFD (Kumar et al. 2005, 2008). This altered behaviour of stomata is expected to have some exclusive advantage in the environment of HA. HA locations may develop high leaf temperature on a clear summer day (Larcher et al. 2010). The higher stomatal conductance observed for many plants at HA, which could allow sufficient influx of CO_2, would experience lower leaf temperature due to higher transpiration rates and likely enhance photosynthetic performance under such higher temperature conditions (Vats et al. 2009). Enhanced stomatal conductance, which supports higher CO_2 uptake, often reduces water use efficiency of plants and may not be a favoured trait under water-limiting conditions. The trait has significance under waterlogged conditions and thus for selection and breeding purposes.

Further on, high irradiance at low temperature and low partial pressure of CO_2 raise the probability of higher rate of photorespiration at HA and consequently enhance higher probability of photo-oxidative damage in plants. Higher oxygenase activity of Rubisco, indicative of enhanced photorespiration, was observed in crops, viz. barley and wheat, and *Rumex nepalensis* grown at high altitude in western Himalaya (Kumar et al. 2006a, b, 2008). Superoxide dismutase (SOD) and ascorbate peroxidase (APX) are the enzymes that could combat such damage (Vyas and Kumar 2005a, b). This has also been related with the winter dormancy in tea (Vyas et al. 2007). Photorespiratory cycle is one of the different adaptive strategies to provide strong electron sink and protect leaves from photo-oxidative damage at HA environment (Streb et al. 1998). An ability of plants at HA was reported (Kumar et al. 2006a, b, 2008) whereby plants overexpressed enzyme, PEPCase (phosphoenolpyruvate carboxylase) activity and operated GS:GOGAT (glutamine synthetase/glutamine 2-oxoglutarate aminotransferase) pathway to recapture carbon and nitrogen lost through the process of photorespiration.

The probability of photo-oxidative gets further enhanced at low or high temperature (Beery and Bkorkman 1980). Per 10 °C drop in temperature, activities of most enzymes decrease by a factor of approximately 2 (Leegood and Edwards 1996). At extreme temperature conditions, reactive oxygen species (ROS), those that harbour the potential of cellular damage and impede repair process, are produced at a much greater rate (Wise 1995; Apel and Hirt 2004; Murata et al. 2007). Similarly higher radiation load can generate excess production of ROS in chloroplasts and lead to DNA, carbohydrate and protein degradation and lipid peroxidation and promote photoinhibition (Moller et al. 2007; Takahashi and Murata 2008). To circumvent the photo-oxidative damage, plants at HA produce more antioxidants (Wildi and Lutz 1996; Streb and Feierabend 1999). Details on imposition of oxidative stress and adaptive strategies is discussed at length in Sect. 10.4.

10.3.3 Response to Temperature

Plant species response to temperature in mountain-ecosystem is one of the most studied one but has been less studied in context to the Himalaya. The impact of low temperature on HA plants can be seen in having lower temperature optima for photosynthesis (Billings and Mooney 1968; Friend and Woodward 1990). Körner and Diemer (1987) reported that leaf grown at 2600 m maintained 50 % of photosynthetic capacities around 4 °C, but those at 600 m altitude could maintain the same around 8 °C. Low temperature is associated with reduced rates of tissue expansion, and consequently greater amount of photosynthetic machinery per unit leaf area, and may therefore influence plant's photosynthetic efficiency (Friend and Woodward 1990). Rubisco activity is a major component that affects photosynthesis, and its activity in alpine population of *Oxyria digyna* was reported to increase when grown at low rather than higher temperature regimes (Chabot et al. 1972). Interestingly, a similar response in the arctic population of the same species was not detected, thus indicating that temperature alone may not be responsible to alter Rubisco activity.

Low temperature is known to reduce metabolism and growth and may cause cold injuries or death of tissues. Interestingly, most alpine plants are adapted to survive and many able to grow under sub-zero temperatures. Some of the important adaptive responses to low temperature are cold acclimation, frost hardiness and deep supercooling whereby suppression of ice formation is accomplished by accumulating certain sugars and proteins. Arctic plants also experience similar chilling conditions, but HA alpine plants need to adapt to high temperature as well. Studies have shown that arctic plants have the heat tolerance limit of to be 5 K lower (Neuner et al. 2000). The mechanism of heat tolerances has not been well studied at molecular level in HA plants.

Plants dwelling in HA locations generally have genetic system for survival and growth at low temperature. Studies on a high-altitude species, *Rheum australe*, showed an importance of mitogen-activated protein kinase pathway for survival under low-temperature conditions (Ghawana et al. 2010). Another interesting species in cold desert of high altitude is *Caragana jubata*. An analysis of low-temperature-responsive genes (QM) suggests a strong upregulation of these genes in less than 30 min of low-temperature exposure to the plants (Bhardwaj et al. 2010). Yet another identified gene was *lipoxygenase* that responded not only to low temperature but also to abscisic acid, methyl jasmonate and salicylic acid. Of various cues, methyl jasmonate caused the strongest upregulation of low-temperature-responsive gene which implies the importance of jasmonate signalling pathway in low-temperature tolerance (Bhardwaj et al. 2011a, b). Novel gene capable of strong upregulation within 15 min of exposure to low temperature has been reported (Bhardwaj et al. 2012). Genes encoding chaperones and those associated with growth and development dominated at low temperature in *C. jubata*. Expression of 11 late embryogenesis abundance protein genes (*LEA*s) was also reported by us in response to low temperature. Some of these genes have been shown to have constitutive expression, whereas others were overexpressed within 3 h of exposure to low

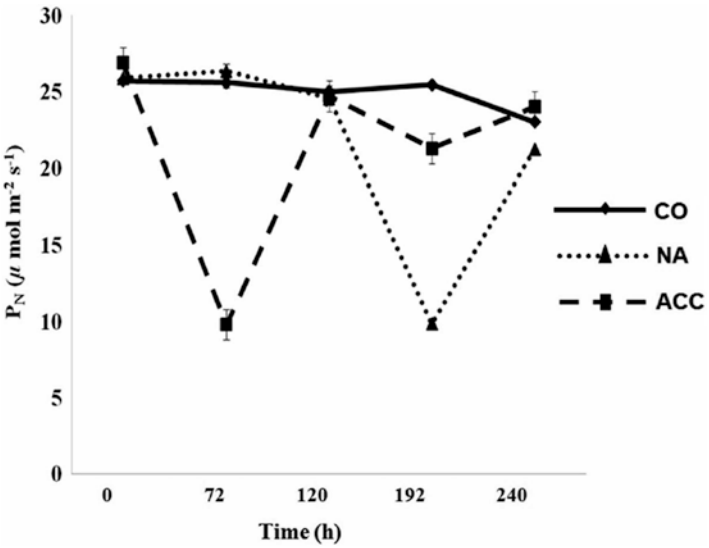

Fig. 10.2 Low-temperature-mediated photosynthetic acclimation in *Caragana jubata* (Reproduced from Bhardwaj et al. 2013; this is an open-access article distributed under the terms of the Creative Commons Attribution License and permits unrestricted use, distribution and reproduction in any medium). Net photosynthetic rates (P_N) were measured in control (CO, X), non-acclimated (NA, m) and acclimated (ACC) plants. CO plants were exposed to 25 °C throughout the experiment. NA plants were initially exposed to 25 °C for 120 h followed by exposure to low temperature (4 °C) up to 192 h for the next duration of 72 h; thereafter, plants were exposed to 25 °C again. ACC plants were exposed to a cycle of 4 °C, 25 °C, 4 °C and 25 °C, at 0 h, 72 h, 120 h and 192 h, respectively

temperature. In response to low temperature, only three *LEA*s exhibited upregulation (*Arabidopsis* and soybean), whereas expression leads to upregulation of only one *LEA* in rice. Simultaneous upregulation of 11 *LEAs* was an exceptional result and possibly explained the performance of the species under extreme environmental conditions at HA.

Apart from expressing chaperons, *C. jubata* upregulated the genes associated with growth and development at low temperature such as *indoleacetic acid-inducible protein*, *auxin-responsive factor 7*, *MYB transcription factor 133*, *cold acclimation responsive2* and *cold acclimation specific*. Corresponding gene homologues did not exhibit the similar trend of gene expression in response to low temperature in low-altitude specific species such as *Arabidopsis thaliana*, *Oryza sativa* and *Glycine max*. Apart from quick modulation of gene expression to external cues, the species also exhibited rapid physiological acclimation as compared to other species of lower altitude such as *Arabidopsis* (Gilmour et al. 1988) and *Medicago truncatula* (Pennycooke et al. 2008). Of significance was the ability of *C. jubata* to maintain P_N at low temperature upon acclimation (Fig. 10.2) and also minimize membrane damage at freezing temperatures. These unique molecular and physiological adaptations permit *C. jubata* to inhabit cold desert environment of HA in the Himalaya (Bhardwaj et al. 2013).

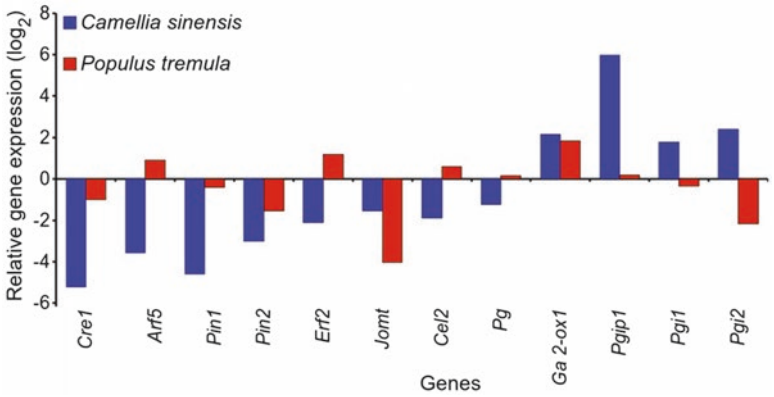

Fig. 10.3 Comparative analysis of expression of various genes associated with leaf abscission in *Camellia sinensis* and *Populus tremula* (Reproduced from Paul et al. 2014; this is an open-access article distributed under the terms of the Creative Commons Attribution License and permits unrestricted use, distribution and reproduction in any medium). Gene expression of *C. sinensis* was based upon reads per kilobase per million (RPKM; during winter dormancy as compared to the period of active growth) values, whereas gene expression for *P. tremula* was based on microarray data [during autumn senescence as compared to the period of active growth (before onset of senescence)] published by Andersson et al. (2004)

Tea (*Camellia sinensis*) plantations make an important component of tropical/temperate landscape in parts of western Himalaya. Our studies in tea identified the candidate genes such as *QM-like protein gene* (Singh et al. 2009a) and *histone H3* (Singh et al. 2009b) and also the gene networks modulated during winters in relation to temperature and hormone responses (Paul and Kumar 2011). Genes responsible for cell cycle or cell division were downregulated, whereas those encoding chaperons and stress-inducible genes were upregulated during winters. Interestingly, genes that have a role in generating cryoprotectants as well as source of energy, e.g. *β-amylase* and *lipase* (Beeveers 1961; Schrader et al. 2004; Ruttink et al. 2007), were also upregulated during winters. The low-temperature-mediated change in gene expression was mimicked by abscisic acid, whereas gibberellic acid mimicked the gene expression modulated by high temperature (Paul and Kumar 2011). An analysis of transcriptome of tea leaves showed that the species also inhibits leaf abscission during winters by modulating the senescence-related processes in contrast to deciduous tree species, *Populus tremula*, that offers tea as an evergreen habit (Fig. 10.3) (Paul et al. 2014). Also, this study explained the molecular basis of evergreen habit of tea tree as compared to deciduous tree *P. tremula*.

10.3.4 Drought Stress

Of other important environmental cues, drought is the one that may influence plant performance by impairing membrane integrity, photosynthetic activity, growth and development processes. Plants develop varied strategies to tolerate drought stress,

e.g. by minimizing water loss (Ruiz-Sánchez et al. 2007), increase soluble sugars/proline content or develop efficient antioxidative systems (Ren et al. 2007; Xiao et al. 2008; Xu et al. 2008). Interestingly, drought stress adds to heat and/or salt stress. Muoki et al. (2012) highlighted the molecular response to these varied stresses in tea. Tea deploys chaperones to protect against drought-related damages and includes genes such as *thaumatin-like protein*, *chitinase* and *late embryogenesis abundant protein3*. These genes exhibited the similar response to drought, osmotic, salt and heat stress (Muoki et al. 2012). Hence, they could be useful targets to generate 'stress-proof' tea. It was interesting to note that the rate of drought imposition was important and also how the stress was imposed. Drought stress and the osmotic stress induced by polyethylene glycol produced very different results, and the data need to be interpreted very carefully (Muoki et al. 2012).

In order to understand the desiccation-responsive processes in the embryo of tea seeds, a proteomic approach was also followed (Chen et al. 2011). Data suggested upregulation of defence response, metabolism and redox status under desiccation. Higher accumulation of ROS, in want of inefficient scavenging by the antioxidant machinery, impacted seed viability. Therefore, production of ROS needs to be managed to minimize desiccation and improve germination rates.

10.4 Oxidative Stress and Adaptive Strategies

Low temperature, high irradiance and drought are known to impose oxidative stress in plants; incidentally, these are also the prevailing cues in mountains. Studies on several clones of tea (*Camellia sinensis*) showed that as the atmospheric temperature declined, P_N also declined along with increase in chlorophyll fluorescence (Joshi and Palni 1998; Joshi et al. 2000; Hazra and Kumar 2002; Vyas et al. 2007). ROS, estimated as superoxide radical, also increased at low-temperature conditions in all the tea clones. The decrease in the rates of photosynthesis and increase in chlorophyll fluorescence in all the clones with concurrent increase in the ROS suggested imposition of oxidative stress in tea during winter months. A strong negative correlation was obtained between the levels of free radicals and the rate of bud growth. An efficient scavenging of ROS appeared a desirable feature in tea since lowered ROS accumulations during winter months were associated with reduced period of winter dormancy (Vyas et al. 2007). Of the various enzymes of antioxidative pathway, glutathione reductase and Mn-superoxide dismutase (Vyas and Kumar 2005b) were identified to be critical.

In the absence of efficient scavenging, superoxide radical and hydrogen peroxide could quite be deleterious. Therefore, the above studies could offer clues that manage oxidative stress for optimal plant performance under stress conditions. Therefore, scavenging of ROS was studied in an altitude plant *Potentilla atrosanguinea* that grows at 4000–4500 m (Sahoo et al. 2001). The plant invariably experienced high radiations and low temperature, the cues that generate superoxide radical within plant cells (Allen 1995). Since superoxide radical is a toxic species and generates

hydroxyl ion which is yet another toxic species, operation of an efficient process for scavenging O_2^- in the species was envisioned. An analysis of antioxidant pathway in *P. atrosanguinea* yielded a superoxide dismutase (SOD), the first enzyme of the pathway that fairly tolerated autoclaving and exhibited activity even at minus temperatures (Kumar et al. 2002, 2006a, b; Sahoo 2004). Crystal structure of the SOD suggested it to be one of the most compact SODs since the gap-volume index was the minimum.

This *SOD* was evaluated to study the effect of its expression in arabidopsis and potato (*Solanum tuberosum* ssp. *tuberosum* L. cv. Kufri Sutlej) under stress conditions (Gill et al. 2010a, b; Pal et al. 2013). SOD was found to improve lignifications of the conducting tissue that improved plant performance under stress conditions (Gill et al. 2010a, b). It was postulated that the hydrogen peroxide generated by SOD-mediated reaction served as a substrate and signal of genes for lignin biosynthesis. Indeed, critical genes of lignin biosynthesis, *phenylalanine ammonia-lyase* and *peroxidase*, were upregulated in the transgenic *Arabidopsis*. It remains to be seen if lignification of vascular tissues could be used as a strategy to improve stress tolerance in plants.

Increase in plant temperature by 10–20 °C higher than the ambient during drought stress is inevitable due to reduction in transpiration cooling. This is accompanied by the accumulation of superoxide radical. Therefore, there is a need of a SOD that shows stability at higher temperature for longer time period. Particularly for such situations, a thermostable SOD was engineered by replacing a free cysteine with alanine at position 95 to avoid unwanted disulphide bond formation between the two SOD molecules. Such a mutation enhanced thermostability of SOD by twofold, and also the enzyme was a kinetically stable protein (Fig. 10.4) (Kumar et al. 2012a, b, c). The use of this engineered SOD will find its place in developing transgenic plants for imparting tolerance at least for high temperature and drought stress.

10.5 Secondary Metabolites and Altitude

Himalayan region is rich in plants having medicinal and aromatic properties due to the presence of a wide range of secondary metabolites. Composition as well as quantity of secondary metabolites is affected by environmental factors such as radiations, temperature and drought¸ apart from growth stage of the plant, nutritional status or microbial infestation (Gairola et al. 2010; Harborne 1982; Kainulainen et al. 1998; Kainulainen et al. 2000; Jochum et al. 2007). Carbon allocation under stress conditions alters metabolite flux, gene expression and transporter response which favours synthesis of secondary metabolites (Mooney et al. 1991). Radiations, particularly UV, increase with increase in altitude. UV-B radiation has been shown to modify secondary metabolite composition of plants such as flavonoids (Ganzera et al. 2008; Zidorn 2010; Jaakola et al. 2004; Winkel-Shirley 2002). Phenolic acids in *Matricaria chamomilla* (Ganzera et al. 2008) and *Arnica montana* (Spitaler et al.

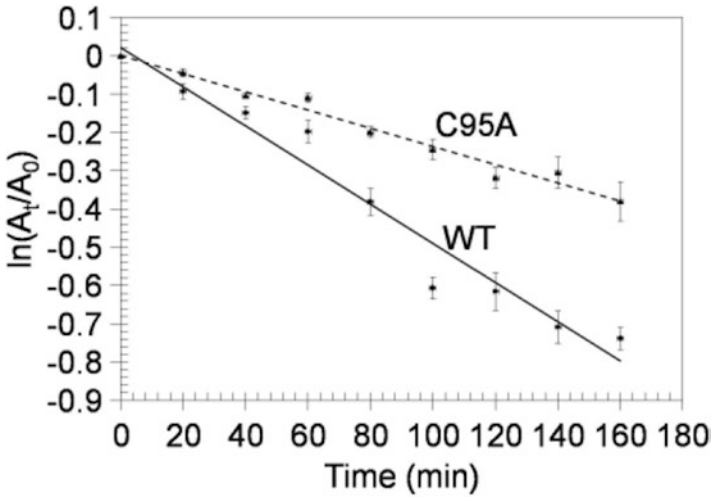

Fig. 10.4 An engineered thermostable superoxide dismutase developed for plant species experiencing heat stress and the drought stress (that usually accompanies high temperature). The first-order thermal inactivation kinetics suggests C95A (where cysteine at position 95 was substituted with alanine) to be more thermostable than the WT (wild type) (Reproduced from: Kumar et al. (2012a, b, c; Singh et al. 2009a, b, c; this is an open-access article distributed under the terms of the Creative Commons Attribution License and permits unrestricted use, distribution and reproduction in any medium)

2008) have also been reported to increase with altitude. These compounds strongly absorb UV (Winkel-Shirley 2002), and an increase in UV-B absorbing and antioxidant phenolic compounds in plants might provide a protective role (Spitaler et al. 2008). Phenolics, particularly flavonoids, are also known to modify growth and development of plants (Rani et al. 2011) and act as allelochemicals (Kim et al. 2005). Though there are no intense systematic studies on the effect of altitude on composition of secondary metabolites, work has been initiated to understand the molecular basis of regulation of target metabolites as will be discussed below.

Picrorhiza kurroa Royle ex Benth., distributed at ~4000 m above mean sea level in the Himalaya, is one of the high-altitude species which is largely used as a hepatoprotectant (Singh et al. 2011). The species due to its heavy extraction from the wild needs conservation and cultivation to meet the rising demand of industry. Hepatoprotective trait of *P. kurroa* is attributed to the presence of picrosides which are glycosidic derivative of iridoid. The iridoid moiety is composed of iridane skeleton, which can be synthesized by mevalonate (MVA) and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways. Phenylpropanoid pathway supplies other moieties such as cinnamate for the biosynthesis of picrosides (Fig. 10.5). Low temperature (15 °C) was found to favour picroside biosynthesis/accumulation, which was due to modulation of the genes involved in the biosynthesis of the moiety (Kawoosa et al. 2010). High-throughput de novo transcriptome analysis showed temperature-mediated transcriptome alterations wherein transcripts for redox, glycogen biosynthetic

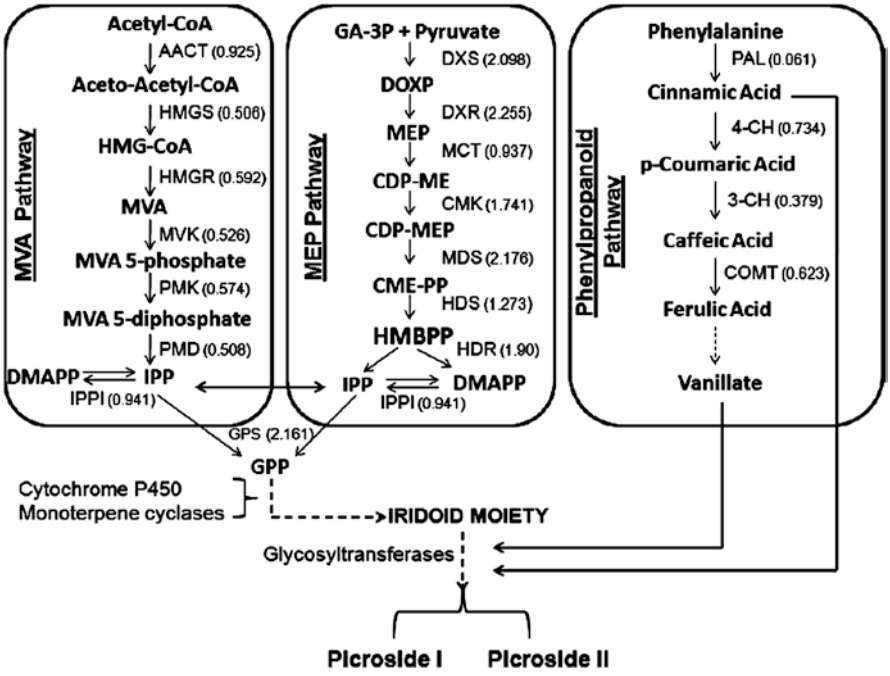

Fig. 10.5 Picroside biosynthetic pathway in *P. kurroa* (Reproduced from: Gahlan et al. 2012; an open-access article distributed under the terms of the Creative Commons Attribution License and permits unrestricted use, distribution and reproduction in any medium). Picrosides are iridoid glycosides derived from cyclization of geranyl pyrophosphate (GPP) to iridoid moiety. Glucose and cinnamate/vanillate convert iridoid into picroside I and picroside II. These steps involve series of hydroxylation and glycosylation reactions catalysed by cytochrome P450 and glycosyltransferases. GPP can be derived from mevalonate (MVA) or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Enzymes of MVA pathway are as follows: *AACT* acetyl-CoA acetyltransferase, *HMGS* 3-hydroxy-3-methylglutaryl-CoA synthase, *HMGR* 3-hydroxy-3-methylglutaryl-coenzyme A reductase, *MVK* mevalonate kinase, *PMK* phosphomevalonate kinase, and *PMD* diphosphomevalonate decarboxylase. Enzymes of MEP pathway are DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; MDS, 2-C-methyl-D-erythritol 2,4- cyclodiphosphate synthase; HDS, 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; and HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase. Isopentenyl pyrophosphate isomerase (IPPI) catalyses the isomerisation of dimethylallyl pyrophosphate (DMAPP) to IPP, whereas conversion of IPP to geranyl pyrophosphate (GPP) is catalysed by geranyl pyrophosphate synthase (GPS). Enzymes of phenylpropanoid pathway involved in biosynthesis of cinnamate are PAL, phenylalanine ammonia-lyase; 4-CH, cinnamic acid 4 hydroxylase; 3-CH, p-coumarate 3-hydroxylase; and COMT, caffeoyl-CoA 3-O methyltransferase. Solid arrows indicate known steps, whereas broken arrows represent unknown intermediates and enzymes. Numerals in parenthesis indicate fold change in gene expression at 15 °C as compared to 25 °C based on reads per exon kilobase per million (RPKM) values

process, various genes of the biosynthetic pathway and purine transmembrane transporter activity were upregulated at low temperature, whereas transcripts associated with the response to biotic stimulus, phytosteroid metabolic process and brassinosteroid and lipid catabolic process were solely expressed at high temperature (25 °C).

Apart from temperature, picroside accumulation was also favoured by light wherein various genes of the picrosides biosynthetic pathways were also modulated (Gahlan et al. 2012). Effect of light was found to be independent of sugar levels. Promoter analysis of these genes showed that GATA and SORLIP are functional *cis*-acting elements that regulated expression of the genes of the pathway (Kawoosa et al. 2014).

Yet another medicinally important species at high altitude is *Sinopodophyllum hexandrum* (Royle) T.S. Ying, which grows at elevations ranging between 2500 and 4500 m above mean sea level. Rhizome of the species yields a cytotoxic aryltetralin lignan podophyllotoxin. Some of its derivatives such as VP-16 and VM-26 possess anticancerous activities, and hence podophyllotoxin is an important metabolite of medicinal importance. Interestingly, this species shows good growth at low temperature of 15 °C as compared to higher temperatures of up to 35 °C (Kumari et al. 2014). Deep sequencing of transcriptome revealed that the processes for growth and development were favoured at 15 °C as compared to those at 25 °C (Fig. 10.6) (Kumari et al. 2014). Interestingly, a decreasing trend of podophyllotoxin accumulation was noticed at 25 °C. Gene expression data supported the physiological and biochemical observations. Also, analysis of various genes of podophyllotoxin biosynthetic pathway supported the data on podophyllotoxin content. Interestingly genes associated with stress response were overexpressed at 25 °C (Fig. 10.6) suggesting this temperature to impose stress on the species, an observation that was also recorded for *P. kurroa* (Gahlan et al. 2012).

Arnebia euchroma (Royle) Johnston (family Boraginaceae) is another medicinally important plant species of high altitude whose roots yield red-coloured naphthoquinone pigments shikonin and its derivatives. These pigments are used in several pharmaceutical and cosmetic preparations (Kim et al. 2001; Papageorgiou et al. 1999). In systematic studies using low and high shikonin-producing systems and appropriate inhibitors (mevinolin of MVA, fosmidomycin of MEP) of the two pathways of geranyl pyrophosphate (GPP, basic precursors), it was shown that the species used MVA pathway for the biosynthesis of GPP. Importantly, *3-hydroxy-3-methylglutaryl-CoA reductase*, *p-hydroxybenzoate-m-geranyltransferase* and all the genes of phenylpropanoid pathways were found to play critical role in shikonin biosynthesis (Singh et al. 2010).

Tea is yet another species of commercial importance that is cultivated in lower (Kangra) region in the western Himalaya. Tea quality is determined by epicatechin (EC) and its derivatives epicatechin gallate, epigallocatechin and epigallocatechin gallate, which are collectively known as epicatechins (ECs). ECs have medicinal properties as well. ECs are synthesized by phenylpropanoid, and the flavonoid biosynthetic pathways and various genes involved in the pathway have been cloned and analysed (Singh et al. 2009a, b, c; Rani et al. 2012). ECs were generally found to decrease by external cues such as drought, and this was attributed to downregulation of various genes of the pathway (Rani et al. 2012).

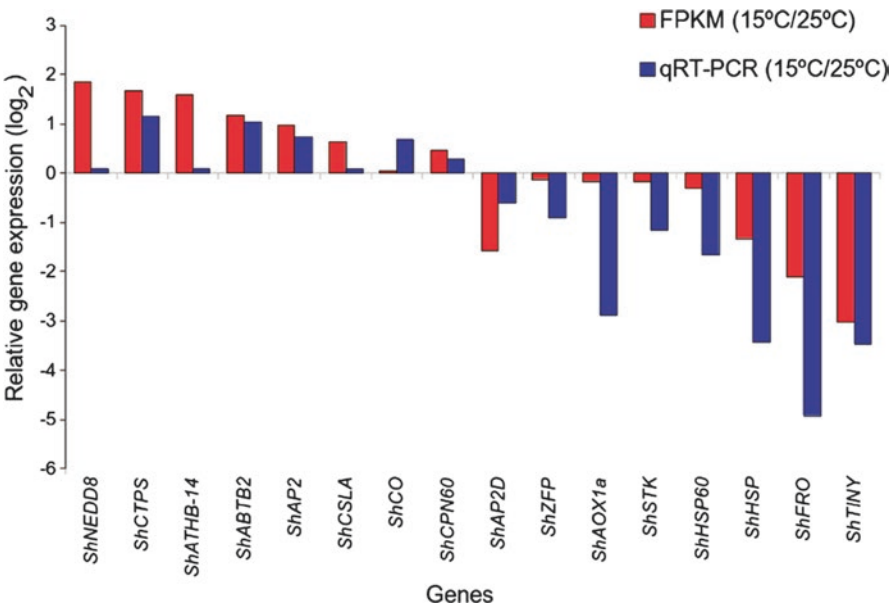

Fig. 10.6 Relative expression of sixteen genes associated with growth and development and stress response at 15 °C as compared to those at 25 °C based upon the data obtained by fragments per kilobase of exon per million fragments mapped (FPKM) values and validated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Name of each gene starts with a prefix Sh that stands for *Sinopodophyllum hexandrum*. Correlation coefficient of FPKM and qRT-PCR was 0.811 (*p*-value = 7.86 e-05) (Reproduced from: Kumari et al. (2014); this is an open-access article distributed under the terms of the Creative Commons Attribution License and permits unrestricted use, distribution and reproduction in any medium)

In line with work on tea, the molecular basis of steviol glycosides was also studied in stevia (*Stevia rebaudiana*) that yields diterpenoid steviol glycosides (SGs). SGs are about 300 times sweeter than sugar and are used as non-calorific sweetener in various countries. Efforts have been made to understand the molecular basis of steviol glycoside biosynthetic pathway in *Stevia rebaudiana*, wherein *1-deoxy*-D-*xylulose 5-phosphate reductoisomerase* and *kaurene oxidase* were identified as regulatory genes (Kumar et al. 2012a, b, c). Of various genes of the pathway, gibberellin (GA_3) upregulated the expression of *4-*(*cytidine 5′ diphospho*)*-2-C-methyl*-D-*erythritol* (*MCT*), 4-(*cytidine 5′ diphospho*)*-2-C-methyl*-D-*erythritol kinase* (*CMK*), *2-C-methyl*-D-*erythritol 2,4-cyclodiphosphate synthase* (*MDS*) and glucosyltransferases SrUGT74G1, whereas methyl jasmonate and kinetin downregulated the expression of all the fifteen genes of the pathway. *MDS* of *S. rebaudiana* was downregulated by darkness as well as indole 3-acetic acid, whereas abscisic acid did not affect its expression (Kumar and Kumar 2013). (E)-*4-hydroxy-3-methylbut-2-enyl diphosphate reductase* (*HDR*), yet another gene of the SG biosynthetic pathway, exhibited diurnal variation in expression. Promoter analysis followed by electrophoretic mobility shift assay suggested the involvement of GATA box in light-mediated gene regulation of SrHDR in stevia (Kumar and Kumar 2013).

Apart from the species discussed above, Himalayas are home to plant species of commercial importance such as *Aconitum heterophyllum*, *Bergenia stracheyi*, *Dactylorhiza hatagirea*, *Datisca cannabina*, *Ephedra gerardiana*, *Ferula jaeschkeana*, *Geranium wallichianum*, *Hippophae rhamnoides*, *Hyssopus officinalis*, *Inula racemosa*, *Juniperus polycarpos*, *Jurinea dolomiaea*, *Polygonatum verticillatum*, *Rheum australe*, *Rhododendron anthopogon*, *Saussurea gossypiphora* and *Viola biflora* (moef.nic.in/downloads/public-information/hfri-300,611.pdf). It will be interesting to study how these metabolites are modulated by environmental cues and do these offer any adaptive advantage under stress.

10.6 Actionable Points

Future research in the Himalaya needs renewed attention. Various studies that could be undertaken on Himalayan ecosystem would be as follows:

1. Study the effect of various radiations (e.g. UV, red/far-red) on plant adaption.
2. Study the effect of soil types on plant performance. For example, the Himalaya originated from sea and is expected to have sea soil bed with a very different nutrient composition. Does this tender uniqueness to the plants?
3. Experiments on integrated effect of various cues on plant performance will be important (e.g. low partial pressure of gases coupled with variable temperature and radiations).
4. In order to establish a cause and effect relationship for secondary metabolites, for example, are these metabolites a result of stress on plants due to altered metabolism or does it have evolutionary significance in offering adaptive advantage to plants?
5. Deployment of omic tools such as metabolomics, ionomics, proteomics and glycomics will be rewarding to decipher unanswered questions on plant adaption in relation to altitude. It is not well known as to how the success of an organism is achieved in the harsh environment of the Himalaya. what are the biological mechanisms that influence the ecologically important adaptive traits, and how these traits affect evolutionary fitness in the ecosystem. This calls for multipronged approach integrating processes at molecular, cellular, organismal, population and ecological level. Strength of ecology, mathematical modelling and population genetics should also be integrated.
6. Mountain ecosystem is vulnerable to climate change. Particular attention is to be paid to the species which are at the border of their spatial distribution, e.g. at higher elevations. Any unfavourable changes in climatic conditions could lead to extinction of the species from the ecosystem. Therefore, appropriate studies on climate change and Himalayan ecosystem should be carefully designed, and appropriate infrastructure should be laid down to answer critical questions of national and global relevance.

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Chapter 11
Drought-Associated MicroRNAs in Plants: Characterization and Functions

Priyanka Soni and Malik Zainul Abdin

Abstract Drought is the main abiotic stress aspect that limits plant growth and productivity worldwide. Plants respond to these conditions by employing gene regulation mechanisms. MicroRNAs (miRNAs) are small, non-coding RNAs, which have newly arisen as important regulators in development, gene regulation, and stress tolerance specifically at the post-transcriptional level in plants. Understanding of the development of miRNAs and miRNA-mediated gene regulation has been made possible by identification of miRNAs in a huge number of important plant species. This chapter provides a short outline on miRNAs, biogenesis of miRNAs, and detection techniques for miRNAs, with a major emphasis on several differentially regulated plant miRNAs under water stress conditions.

11.1 Introduction

During the past few years a number of studies have indicated the principal role of microRNAs (miRNAs) in response to abiotic plant stresses, including drought. miRNAs were first recognized in *Caenorhabditis elegans* by genetic mapping (Lee et al. 1993; Wightman et al. 1993). These are small (approximately 20–24 nucleotides in size), endogenous, non-coding RNA regulatory molecules, which control the gene expression involved in numerous vital metabolic processes. Modulation of gene expression by miRNAs is achieved by binding at the 3′ untranslated regions (3′-UTR) of specific mRNAs, followed by RNA silencing and post-transcriptional regulation.

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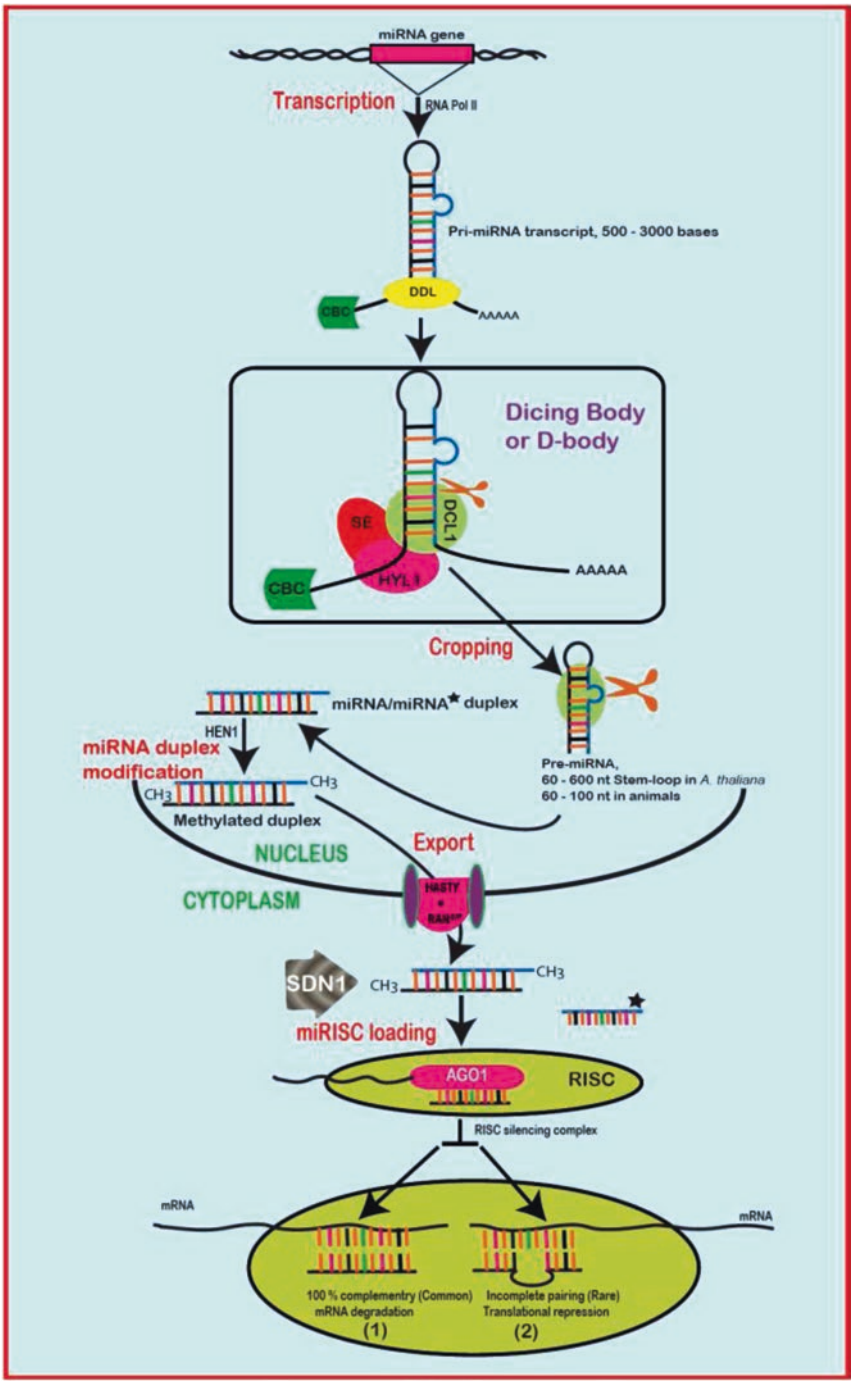
11.1.1 Features of miRNAs

miRNAs have numerous specific characteristics, such as the fact that they fit into identical family—suggesting their simultaneous evolution—and are determined as polycistronic transcripts. Great conservancy in miRNA sequences among diverse organisms has been observed, which shows their involvement in specific cellular functions (Berezikov et al. 2005). Lastly, miRNAs control several mRNAs simultaneously rather than targeting a specific mRNA at a time.

11.1.2 miRNA Biogenesis

In contrast to animal miRNAs, transcription of plant miRNAs is completed in the nucleus by RNA polymerase II, and they are 5′ capped and 3′ polyadenylated (RNA pol II) (Cui et al. 2009; Lee et al. 2004) (Fig. 11.1). Newly formed transcripts are called primary miRNAs (pri-miRNAs), which can be a few kilobases extended and stretched with stem-loop structures. The development of mature miRNA from pri-miRNA is a sequential procedure including the role of Dicer-like protein (DCL1), which is expressed simply in the plant cell nucleus. In plants, pre-miRNA is cleaved by DCL1 in the nucleus, whereas in animals, pre-miRNA is first transferred from the nucleus and Dicer enzyme performs the cleavage reaction in the cytoplasm (Kim, 2005). Some other important proteins have been found to be essential in *Arabidopsis*—Hyponastic Leaves1 (HYL1), Zn-finger protein SERRATE (SE), and RNase III Dicer-Like1 (DCL1)—which work collectively in nuclear processing centers, called D-bodies or SmD3/SmB-bodieusing, for the correct processing of precursor miRNA (pre-miRNA) (Kurihara et al. 2006). Before transport from the plant nucleus, miRNA:miRNA* duplexes are characterized by 3′ overhangs and are methylated by a RNA methyltransferase protein called Hua-Enhancer1 (HEN1)

Fig. 11.1 Biogenesis of miRNAs in plants. The primary transcript (primiRNA) of the miRNA gene is transcribed by RNA polymerase II (pol II). DAWDLE (DDL), an RNA-binding protein, alleviates the stem-loop for adaptation into D-bodies (nuclear processing centers) and as well cooperates with Dicer-like-1 (DCL1) in order to support stem-loop precursor identification. The mature miRNA duplex is edited from the primiRNA (miRNA/miRNA*), where miRNA is the guide strand (in *black*) and miRNA* is the degraded strand (in *blue*). The addition of a methyl group is done by the S-adenosyl methionine (SAM)-dependent methyltranferase enzyme. HUA ENHANCER1 (HEN1) stops the duplex from being ruined by the small RNA degrading nuclease (SDN). C2H2-zinc finger protein SERRATE (SE), the double-stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1), and nuclear cap binding protein (CBC) also play important roles for right processing of precursor miRNA. HASTY transfers the miRNA/miRNA* from the nucleus to the cytoplasm. The guide miRNA strand is integrated into RNA-induced silencing complex (RISC) to perform target mRNA cleavage followed by translational repression (Cui et al. 2009; Lee et al. 2002)


miRNA gene
Transcription
RNA Pol II
Pri-miRNA transcript, 500 - 3000 bases
DDL
CBC
AAAAA
Dicing Body
or D-body
SE
DCL1
HYL1
CBC
AAAAA
Cropping
miRNA/miRNA* duplex
HEN1
miRNA duplex
modification
CH3
CH3
Methylated duplex
Pre-miRNA,
60 - 600 nt Stem-loop in A. thaliana
60 - 100 nt in animals
NUCLEUS
Export
CYTOPLASM
HASTY
SDN1
CH3
CH3
miRISC loading
AGO1
RISC
RISC silencing complex
mRNA
mRNA
100 % complementry (Common)
mRNA degradation
(1)
Incomplete pairing (Rare)
Translational repression
(2)

(Elbashir et al. 2001), whereas in animals the miRNA:miRNA* duplex is managed by the RNase III enzyme Drosha in collaboration with Pasha (Lee et al. 2003). This duplex is then further transferred from the nucleus to the cytoplasm with the help of a protein called Hasty (HST), an Exportin 5 homolog, in animals, in association with RAN1-GTP (Telfer and Poething 1998; Bollman et al. 2003). The protein S-adenosyl methionine-dependent methyltransferase-HUA ENHANCER1 (HEN1) methylates RNAs at the 3′ terminal nucleotides and stabilizes the mature miRNA duplexes in order to prevent them from degradation in the cytoplasm (Yu et al. 2005). The miRNA:miRNA* duplex then disassembles, and mature miRNA is integrated into RNA-induced silencing complex (RISC) and cooperates with AGO protein to control gene expression and perform different cellular tasks, including cleavage of target mRNA, translation silencing, and regulation of the chromatin structure (Khvorova et al. 2003) (Fig. 11.1).

11.2 miRNAs in Drought Stress

Drought is the leading abiotic stress, initiated by a lack of rainfall, soil water deficit, and excessive dehydration (Shukla et al. 2008). In response to a drought situation, plants use tactics to lessen transpiration, save water, and explore molecular mechanisms to retain water. Recently some specific miRNAs have been identified in plants as playing vital roles in water stress, acting as the main elements in regulating genes and stress tolerance (Lelandais-Briere et al. 2009; Li et al. 2011a, b, c; Xu et al. 2010; Zhao et al. 2007). Vasudevan et al. (2007) reported that some miRNAs could up-regulate gene expression in specific cell types and conditions with distinct transcripts and proteins, while, at the same time, some could down-regulate the expression in a particular cell. Furthermore, a single miRNA can perform in both up- and down-regulation; similarly, a single specific gene might come across both regulation commands based on the specific conditions and factors. Recent studies in many crops—including *Arabidopsis thaliana* (Liu et al. 2008), tobacco (Frazier et al. 2011), soybean (Kulcheski et al. 2011), and *Triticum dicoccoides* (Kantar et al. 2011)—have also pointed out the fact that miRNAs are differentially regulated. According to Barrera-Figueroa et al. (2011) the level of expression of an miRNA is species specific. For instance, miR156 was found to be drought up-regulated in *Arabidopsis* and *Triticum dicoccoides* (Eldem et al. 2012) but down-regulated in rice and maize (Zhou et al. 2010). Drought up- or down-regulated miRNAs are both significant for engineering plant drought tolerance. Several water stress–associated miRNAs have been studied in many plant species and are summarized in Table 11.1.

Table 11.1 Common miRNAs related to drought stress response in plants

Plant	miRNA	Target genes and proteins	References
Maize	zma-miR1–9	FDH, IRL, XTH1, ZmLD	Chen et al. (2010)
Populus euphratica	miR123	Cell death domain	Cohen et al. (2010)
Arabidopsis, rice, *Populus euphratica*	miR156	SBP family TFs	Kantar et al. (2010), Li et al. (2011a), Lu et al. (2008)
Arabidopsis	miR157	SBP	Liu et al. (2008)
Arabidopsis, *Nicotiana tabacum*, *Oryza sativa, Panicum virgatum*	miR159	MYB	Alonso-Peral et al. (2012), Frazier et al. (2011), Reyes and Chua (2007), and Shuai et al. (2013)
Phaseolus vulgaris	miR159.2	Chlatrin heavy chain	Arenas-Huertero et al. (2009)
Brachypodium distachyon	miR160	Ca-responsive/WAK receptor	Bertolini et al. (2013)
Maize	zma-miR161	Unknown	Chen et al. (2010)
Populus euphratica, maize, rice	miR162	dcl1	Li et al. (2011) and Zhou et al. (2010)
Brachypodium distachyon	miR164	NAM	Bertolini et al. (2013)
Arabidopsis, barley, soybean, wheat, maize	miR165/miR166	HD-ZIP III TFs	Kantar et al. (2010), Li et al. (2011), Lv et al. (2010), Xin et al. (2011), and Zhang et al. (2011)
Arabidopsis, maize, rice	miR167	ARF6 and ARF8 TFs	Frazier et al. (2011), Li et al. (2010), Lv et al. (2010), and Zhang et al. (2011)
Arabidopsis, rice, *Populus*, wheat	miR168	ago1	Lu et al. (2008) and Xin et al. (2011)
Arabidopsis, rice, tobacco, tomato	miR169	CCAAT-BOX BF, NF subunit Y	Frazier et al. (2011), Zhang et al. (2009), and Zhao et al. (2007)
Arabidopsis, Populus, barley, rice	miR171	SCL TFs	Frazier et al. (2011), Kantar et al. (2010), and Lv et al. (2010)
Arabidopsis, Populus, barley, rice	miR172	AP2 TF, PHAP2A protein (Q9XHD4)	Frazier et al. (2011), Xin et al. (2011), and Zhang et al. (2009)

(continued)

Table 11.1 (continued)

Plant	miRNA	Target genes and proteins	References
Arabidopsis, rice	miR319	TCP family TFs	Liu et al. (2008), Lv et al. (2010), and Zhang et al. 2011
Arabidopsis	miR389a	Unknown	Sunkar and Zhu (2004)
Arabidopsis, rice, wheat	miR393	F-BOX protein, tir1, afb2 and afb genes	Arenas-Huertero et al. (2009), Frazier et al. (2011), Sunkar et al. (2012), Xin et al. (2011), and Zhao et al. (2007)
Arabidopsis, rice, *Brachypodium distachyon*	miR395	AP, APS4/F-box	Bartel 2004, Bertolini et al. 2013, and Zhou et al. 2010
Arabidopsis, barley	miR396	GRF family TFs	Chi et al. (2011), Frazier et al. (2011), and Kantar et al. (2010)
Arabidopsis, maize	miR397b	LAC	Chen et al. (2010) and Sunkar and Zhu (2004)
Medicago truncatula, Solanum, rice	miR398	Cu/Zn superoxide dismutase	Nageshbabu et al. (2013), Trindade et al. (2010), and Kang et al. (2012)
Arabidopsis	miR402	*DML3*	Sunkar and Zhu (2004)
Cowpea	miR403	AGO2	Allen et al. (2005) and Barrera-Figueroa et al. (2011)
Populus, barley	miR408	Plantacyanin, TCP14	Bertolini et al. (2013), Kantar et al. (2010), and Trindade et al. (2010)
Maize	zma-miR446	Unknown	Chen et al. (2010)
Triticum dicoccoides	miR474	Kinesin	Kantar et al. (2011)
Maize	zma-miR479	PDC1	Chen et al. (2010)
Cowpea, soybean, *Glycine max*	miR482	ARA12, zinc finger protein	Barrera-Figueroa et al. (2011), Kulcheski et al. (2011), and Li et al. (2011)
Populus trichocarpa	miR482.2	Unknown	Lu et al. (2008)
Brachypodium distachyon	miR528	XBAT32	Bertolini et al. (2013)

(continued)

Table 11.1 (continued)

Plant	miRNA	Target genes and proteins	References
Populus trichocarpa, maize	zma-miR530	sus2	Chen et al. (2010) and Lu et al. (2008)
Maize	zma-miR776	Unknown	Chen et al. (2010)
Maize	zma-miR782	PDIL5-1	Chen et al. (2010)
Maize	zma-miR815a	NRP1	Chen et al. (2010)
Maize	miR818a	Unknown	Chen et al. (2010)
Maize	zma-miR820	sbp6 domain	Chen et al. (2010)
Triticum aestivum	miR894	Similar to protein phosphatase PP2A-4	Kantar et al. (2011)
Triticum aestivum	miR1432	Phenylalanine tRNA synthaselike	Kantar et al. (2011)
Populus trichocarpa, Triticum aestivum, Triticum dicoccoides	miR1450	Mn superoxide dismutases	Kantar et al. (2011) and Lu et al. (2008)
Glycine max	miR1510	LRR-containing proteins	Kulcheski et al. (2011)
Glycine max	miR1513	F-BOX domain proteins	Kulcheski et al. (2011)
Phaseolus vulgaris	miR1514	PsEMFl-related	Arenas-Huertero et al. (2009)
Triticum aestivum, Triticum dicoccoides	miR1867	Putative protein, DUF1242 superfamily	Kantar et al. (2011)
Oryza sativa	miR2002	Caltractin	Jian et al. (2010)
Oryza sativa	miR2003	HEAT repeat family protein	Jian et al. (2010)
Oryza sativa	miR2005	Nitrate and chloride transporter	Jian et al. (2010)
Oryza sativa	miR2007	DNA binding protein	Jian et al. (2010)
Arabidopsis, Phaseolus vulgaris	miR2118	U1-70K related	Arenas-Huertero et al. (2009)
Phaseolus vulgaris	miR2119	ADH1	Arenas-Huertero et al. (2009)

AP adaptor protein, ADH1 alcohol dehydrogenase, AFB Auxin F-box protein, *ago1* argonaute protein, AP2 adaptor protein, ARA12 Arabidopsis subtilisin-like protease, ARF AUXIN RESPONSE FACTOR, BF binding factor, dcl1 Dicer-Like1, DML3 Demeter-like protein, DUF1242 Domain of unknown function, F-BOX Flagellin Box, FDH Formate Dehydrogenase, GFR growth-regulating factor, *HD-ZIP III TFs* class III homeodomain leucine zipper, *IRL* Isoflavone reductase homolog-like, LAC Laccase, LRR leucine-rich-repetitions, MYB Myeloblastosis, NAM No apical meristem, NF Nuclear factor Y subunit A, NRP1 N-rich protein, PDC1 pyruvate decarboxylase isozyme1,PDIL 5-1protein disulfide isomerase-like, PHAP2A protein Petunia hybrid ap2-like protein, PP2A-4 protein phosphatase 2A isoform 4, SBP SQUAMOSA-promoter binding protein, sbp6 SBP domain binding protein, SCL scarecrow-like, *sus2* sucrose synthase 2, TCP TEOSINTE BRANCHED/CYCLOIDEA/PCF) transcription factor genes, TIR transport inhibitor response, U1-70K U1 small nuclear ribonucleo 170 K protein, WAK receptor Wall-associated kinase receptor, *xth1* XTH1 putative xyloglucan endotransglucosylase/hydrolase protein 1

11.2.1 miR156

miR156 has been revealed to target squamosal promoter binding protein–like (*SPL*) genes (Bertolini et al. 2013). However, in *Arabidopsis*, maize, rice, and wheat it has been detected to be effective for SPB transcription factor miR156 (Barrera-Figueroa et al. 2011; Kantar et al. 2011; Shen et al. 2010). miR156 is one of the important miRNAs that play a significant function in many cellular processes, such as regulation of shoot growth (Nageshbabu et al. 2013; Park et al. 2011). Some researchers have suggested that it also plays a vital role in drought stress (Barrera-Figueroa et al. 2011; Li et al. 2011a, b, c). Barrera-Figueroa et al. (2011) found miR156 to be enhanced in drought stress in cowpea (Barrera-Figueroa et al. 2011) Similarly, research on *Phaseolus vulgaris* (French bean) showed a great increase in expression of miR156 transcript by 1.7-fold in a severe water stress situation (Nageshbabu et al. 2013). In *Brachypodium* also, water stress–induced miR156 was detected in expanding cells, signifying the role of miR156 in monocots under water stress (Bertolini et al. (2013), though in rice it was found to be down-regulated by 2.1-fold in drought stress (Ding et al. 2011).

11.2.2 miR159

Drought-related miR159s have been observed in *Arabidopsis thaliana*, *Nicotiana tabacum*, *Oryza sativa*, *Panicum virgatum*, and *Populus* (Kantar et al. 2010; Ni et al. 2012; Reyes and Chua 2007; Shuai et al. 2013; Sun et al. 2012). In switchgrass, PEG-induced water stress accumulated miR159 and up-regulation was greatest (a 0.9-fold change) under 7.5 % PEG treatment, which directed that miR159 attribute to the adaptation of switchgrass to drought stress (Sun et al. 2012). Similar observations have been made in *Phaseolus vulgaris* (Arenas-Huertero et al. 2009) and *Arabidopsis* (Reyes and Chua, 2007). miR159a facilitates the cleavage of myb33 and myb101 transcripts in *Arabidopsis*, and it has been suggested that it may play a key role in ABA response by directing the degradation of myb mRNAs in *Arabidopsis*.

11.2.3 miR162

Understanding of miR162 expression under water stress is essential, as it adversely regulates the Dicer-like 1 (*DCL1*) gene, which plays an important part in miRNA biogenesis. Barrera-Figueroa et al. (2011) for the first time exposed drought-related miR162 in grapevine. Drought-induced miR162 was also found in French bean, suggesting that it plays a significant role in adaptation to drought stress (Nageshbabu et al. 2013).

11.2.4 miR164

Drought-regulated miR164 was first reported in *Populus* plants by Shuai et al. (2013). Wang et al. (2011) observed down-regulation of miR164 under water deficit in *Medicago truncatula*, while Bertolini et al. (2013) suggested drought-associated up-regulation of miR164 in *Brachypodium distachyon*.

In *Arabidopsis*, expression of five NAM/ATAF/CUC (NAC) proteins—a class of plant-specific transcription factors, which perform crucial functions in growth and stress responses, including cold, drought, and pathogen attack—was suggested to be controlled by miR164 (Guo et al. 2005; Hasson et al. 2011; Koyama et al. 2010). Ferreira et al. (2012) found differential regulation of miR164 in sugarcane under drought at an early stage, indicating its stage-specific role as a stress response that probably supports plant survival in water stress conditions.

11.2.5 miR165/166

miR165/166 are derived from multiple loci in the genome and share similar sequences (Reinhart et al. 2002). They regulate the growth of leaves by targeting transcription of the class III homeodomain leucine zipper (HD-zip) family genes (Bao et al. 2004).

Liu et al. (2008) found drought-responsive miR165 in *Arabidopsis.* Similarly, water stress–regulated miR166 has been observed in cowpea (Barrera-Figueroa et al. 2011; Kantar et al. 2011). miR166a was found to be induced by water stress, whereas miR166b, which belongs to a similar family, was expressively down-regulated in cowpea. This suggests that the miRNA expression levels brought about by drought stress differ, and they might be part of particular stress response paths and roles (Juarez et al. 2004).

11.2.6 miR167

miR167 targets UDP-glucose 6-dehydrogenase, a fundamental enzyme that is involved in cell wall biosynthesis, leaf development, cell expansion, and is expressed in developing tissues (Klinghammer and Tenhaken 2007). Sunkar et al. (2007) reported several stress-regulated miRNAs, including members of miR167, miR319, and miR393. Likewise, in *Arabidopsis*, drought-related miR167 has been recognized (Barrera-Figueroa et al. 2011). Drought-induced up-regulation of miR167 was detected in *Phaseolus vulgaris* (Nageshbabu et al. 2013) and *Brachypodium distachyon* (Bertolini et al. 2013).

11.2.7 miR168

In rice and *Arabidopsis*, miR168 was observed to target AGO1 protein—the core component of RISC—which is essential for miRNA utility and also supports post-transcriptional stabilization of miR168 (Bertolini et al. 2013; Chen et al. 2010); therefore, miR168 is part of negative-feedback regulation for controlling miRNA biogenesis (Vaucheret et al. 2006).

miR168 has been described to be differentially regulated in drought stress (Sire et al. 2009; Zhou et al. 2010), which shows that miRNA expression under water stress can be complicated and depends on diverse parameters. A genome-wide study performed in rice through diverse developmental stages discovered down-regulation of miR168 under drought stress (Zhou et al. 2010), whereas in *Arabidopsis*, drought up-regulated miR168 has been reported (Kruszka et al. 2012).

11.2.8 miR169

miR169 targets nfya 5 mRNA, which encodes a subunit of the nuclear factor Y (NF-Y) transcription factor (Li et al. 2008), and plays an important role in environmental stresses (Kumimoto et al. 2008). miR169 was perceived to be up-regulated in response to dehydration in a number of crops, including rice (Zhao et al. 2007), tomato (Zhang et al. 2011), and tobacco (Frazier et al. 2011), whereas it was reduced as a response to drought stress in *Arabidopsis*, where the expression level of the nfya 5 gene was subsequently intensely increased and contributed to drought resistance (Frazier et al. 2011; Li et al. 2008). Similarly, Bertolini et al. (2013) also detected a reduced level of miR169 transcript in *Brachypodium distachyon* under stress conditions.

11.2.9 miR171

Ma et al. (2014) reported that miR171 targets SCL (scarecrow-like) proteins, which adversely regulate chlorophyll biosynthesis by silencing the expression of the main gene, protochlorophyllide oxidoreductase (*POR*). miR171 was revealed to be linked with water stress in a number of studies (Zhou et al. 2010). Barrera-Figueroa et al. (2011) detected 44 drought-related unique mature miRNAs, including miR171. Water stress–induced miR171 was also described in rice (Jian et al. 2010), and *Arabidopsis* (Shuai et al. 2013), while in *Populus trichocarpa* the expression level of miR171 was slightly decreased during drought stress (Lu et al. 2008b).

11.2.10 miR172

In *Arabidopsis* seedlings, miR172 targets the schnarchzapfen (*SNZ*) gene, an AP2-like transcription factor (Zhang and Pan 2009), which controls the floral pattern and is also recognized to have utility in the shoot apical meristem (Wurschum et al. 2006). Kim et al. (2011) identified miR172 in pepper and potato to be associated with regulation of PHAP2A protein, a transcription factor, which suggests that miRNA172 plays an important role in controlling several genes of plants by upsetting diverse transcription factors.

Nonogaki (2010) revealed that the miR156 and miR172 paths are linked with each other in both *Arabidopsis* and maize. Frazier et al. (2011) detected water stress–induced miR172 after treatment with variable percentages of PEG in tobacco plants, whereas Zhou et al. (2010) found drought-associated down-regulated miR172 in *Oryza sativa.*

11.2.11 miR319

miR319 targets TCP (Teosinte Branched, Cycloidea, promoter binding factors) transcription factors (Rodriguez et al. 2010). miR319 is considerably more accumulated under drought in proliferating cells than in expanding cells of *Brachypodium distachyon* (Bertolini et al. 2013).

Sunkar and Zhu (2004) identified drought-induced transcripts of miR393, miR319, and miR397 in *Arabidopsis*. A genome-wide study in rice revealed water stress up-regulation of miR319 in different developmental stages of the plant, and elevated levels of miR319 might allow the cell to withstand water stress (Zhou et al. 2010).

11.2.12 miR393

It is known that miR393 is a key factor required for auxin homeostasis. In *Arabidopsis*, miR393 targets an auxin receptor, TIR1 (transport inhibitor response 1), which positively controls auxin signaling by endorsing the breakdown of Aux/IAA proteins by the process of ubiquitination (Dharmasiri and Estelle 2002). On the other hand, in *Vigna unguiculata*, miR393 targets TAS3-ARF, which also performs an essential role in auxin signaling and lateral root growth in plants (Barrera-Figueroa et al. 2011). Differential responses of miR393 were observed in different plants, and induction was observed in *Arabidopsis* (Sunkar and Zhu 2004), rice (Zhao et al. 2007), sugarcane (*Saccharum* spp.), *Vigna unguiculata* (Barrera-Figueroa et al. 2011), and *Phaseolus vulgaris* (Arenas-Huertero et al. 2009) (Ferreira et al. 2012). Soybean cultivars showed variable responses of miR393 during a water

stress situation, and an increased level of transcript was presented by sensitive genotypes, whereas tolerant genotypes showed down-regulation in miR393 activity (Kulcheski et al. 2011). As auxin is a regulator of every stage of the plant cycle, understanding of regulation of miR393-controlled *TAAR* gene expression during stress conditions is very important (Kruszka et al. 2012).

11.2.13 miR395

miR395 targets APS4 (sulfate adenylyltransferase), which triggers catalysis of sulfate in the sulfur assimilation pathway. In addition, miR395 controls the passage of sulfate in leaves (Bertolini et al. 2013; Liang and Yu 2010). In rice, miR395 was observed to be up-regulated (Zhou et al. 2010), whereas in tobacco, miR395 was found to be suppressed under water stress (Shuai et al. 2013). Recently Liang and Yu (2010) established in *Arabidopsis thaliana* that miR395 is critical for sulfate homeostasis by controlling sulfate uptake, transport, and assimilation.

11.2.14 miR396

miR396 has been described to target growth-regulating factor 2 (GRF-2), a main class of transcriptional regulators that control leaf development (Kim et al. 2003; Liang et al. 2013; Rubio-Somoza and Weigel 2011). Liang et al. (2013) stated that miR396 is especially expressed in flowers, but its role in flower development is still uncertain. Liu et al. (2008) recognized drought-related miR396 in *Arabidopsis*. Similarly, Kantar et al. (2010) revealed drought-responsive miR396 in root tissue of barley. Bertolini et al. (2013) and Liu et al. (2008) found up-regulated miR396 in *Brachypodium distachyon* and *Arabidopsis*, respectively.

11.2.15 miR397

Lu et al. (2013) recognized a set of laccase genes as targets of the miRNA miR397 in *Populus trichocarpa*. Laccase (LAC) catalyzes the oxidative polymerization of monolignols in plants and might be involved in stress response (Bertolini et al. 2013; Lu et al. 2013). A number of authors have discovered drought-responsive miR397 (Gray-Mitsumune and Matton 2006; Nageshbabu et al. 2013). In *Panicum virgatum* it was shown to have a function in water stress conditions (Matts et al. 2010; Xie et al. 2010).

miR397 was also described to be associated with water stress in *Solanum* and rice (Kwon et al. 2011; Zhou et al. 2010). Sun et al. (2012) observed dose-dependent differential regulation of miR397 in switchgrass (*Panicum virgatum*) by applying

different concentrations of PEG. Maximum induction of miR397 with a 0.6-fold change was observed at 7.5 % PEG. Liu et al. (2008) reported dehydration-induced miR397 in *Arabidopsis*, whereas contrary results were obtained by Zhou et al. (2010), who detected down-regulation of miR397 in *Oryza sativa* under drought conditions. Consequently, it may be suggested that the same miRNAs identified as responding in water stress in one species may not play an identical role in other species.

11.2.16 miR398

miR398 targets two homologous copper/zinc superoxide dismutases (Cu/Zn SODs) by blocking their translation (Bouché 2010). Cu/Zn SODs protect cells from injury by toxic superoxide radicals (Barrera-Figueroa et al. 2013). Liu et al. (2008) identified Laccases and beta-6-tubulin genes as targets of miR398 in *Arabidopsis*. Drought stress–related miR398 was described in *Solanum* and rice by Nageshbabu et al. (2013). In *Medicago truncatula*, miR398 was found to be improved under drought stress (Kang et al. 2012; Trindade et al. 2010), whereas it was observed to be down-regulated by water stress (Sunkar et al. 2006).

11.2.17 miR402

Kim et al. (2010) identified miR402 as an important role player in seed germination and growth of seedlings in *Arabidopsis* under abiotic stress conditions. miR402 targets REPRESSOR OF SILENCING1 (ROS1)-like protein (At4g34060), recognized as DNA glycosylase and also defined as DEMETER-LIKE protein3 (DML3), which is involved in DNA demethylation (Ortega-Galisteo et al. 2008). The up-regulation of miR402 expression by drought stress has been revealed in *Arabidopsis* (Barrera-Figueroa et al. 2011, 2013; Khraiwesh et al. 2012; Kim et al. 2010; Sunkar and Zhu 2004). Lu et al. (2008) documented minor increases in expression of miR402 transcript.

11.2.18 miR408

In wheat, miR408 targets a chemocyanin-like protein gene (*TaCLP1*), which shows protective function in response to abiotic stress (Feng et al. 2013). Ma et al. (2015) analyzed transgenic plants overexpressing miR408 and concluded that enhanced levels of miR408 transcript increased tolerance of salinity but decreased tolerance of drought stress. Drought-responsive miR408 has been identified in peach (Eldem et al. 2012). miR408 was found to be drought induced in both *Medicago truncatula*

and *Arabidopsis* (Liu et al. 2008; Trindade et al. 2010), whereas it was down-regulated in *Populus trichocarpa* (Lu et al. 2011, Ravet et al. 2011).

11.2.19 miR482

In cowpea, miR482 targets ARA12 (serine-type endopeptidase) (Barrera-Figueroa et al. 2011); however, in maize, CCHC-type zinc finger proteins were anticipated to be targets of miR482 (Zhang et al. 2009). Li et al. (2011) and Kulcheski et al. (2011) reported drought-responsive miR-482 in *Glycine max* and soybean, respectively. Shivaprasad et al. (2012) found differential expression of miR482 in different plant families, including Rutaceae, Solanaceae, and Fabaceae, under adverse conditions.

11.2.20 miR528

Recently miR528 has been recognized as a regulator of the antioxidant defense system under drought conditions in plants. Wei et al. (2009) predicted that *POD* was a target of miR528 and during RT-PCR analysis found that the expression level of *POD* under drought conditions was up-regulated in maize because of the down-regulation of miR528. The increase in POD would enhance ROS-scavenging ability in water deficit stress and relieve the damage triggered by ROS. Thus, miR528 is probably a regulator of antioxidant defense under drought conditions in plants (Ding et al. 2013).

Bertolini et al. (2013) found that miR528 possibly gets involved in the regulation of ethylene degradation through a recognized target, XBAT32 E3 ligase, and the regulation of *XBAT32* by *miR528* might therefore act to maintain overall growth under drought stress. It has been well established that drought stress causes up-regulation of *miR528* in sugarcane (Ferreira et al. 2012) and *Brachypodium* (Bertolini et al. 2013; Budak and Akpinar 2011).

11.2.21 Other miRs

Barrera-Figueroa et al. (2011) investigated the association of some other miR families, such as miR403 and miR828, with water stress. Chen et al. (2010) recognized drought-regulated miRNAs in maize. Regulation of diverse miRNAs under drought stress in different plant species is summarized in Table 11.1.

11.3 Methods for the Study of miRNA Gene Expression

Quantification of expressed miRNA offers crucial information about miRNA-mediated gene regulation in a cell. Many practices have been established for fast, sensitive, and genome-wide detection of miRNAs (Eldem et al. 2013). Some of these quantification methods used for miRNA detection are discussed below.

11.3.1 MicroRNA Profiling Using Microarrays

Microarrays have arisen as an effective, powerful, and widely used tool for genome-wide miRNA expression profiling (Liu et al. 2008; Yin et al. 2008). A microarray is a well-ordered array of DNA probes on a platform that permits the precise binding of genes. The probes most commonly used are complementary DNA (cDNA) or oligonucleotides. A microarray technique for miRNA profiling has been broadly used in plants in a number of studies including abiotic stresses, such as water deficit (Liu et al. 2008; Ding et al. 2011; Li et al. 2011). To be precise, microarrays reflect an innovative technology, easing the quantitative analysis of a number of genes concurrently from a solitary sample. However, there are numerous restrictions related to this technique, such as the insufficient sensitivity of the analysis for recognition of low-abundance genes, and false-positive tests due to non-specific labeling caused by cross-hybridization (Yin et al. 2008). Because of these limits, microarray analysis usually needs additional confirmation by extra subtle and quantifiable assays such as quantitative real-time PCR.

11.3.2 Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR measures an absolute quantity of miRNA in terms of the number of copies of original mRNA based on reverse transcription of miRNA to cDNA, followed by quantitative polymerase chain reaction (qRT-PCR). It is one of the most sensitive, specific, and leading methods; therefore, it has been widely used in concurrent estimation of gene expression in the study of miRNAs (Varkonyi-Gasic et al. 2007). Amplification is started with miRNA-specific and stem-loop/poly (A) primers. Amplified products are measured by either SYBR® Green dye or TaqMan® probe.

SYBR®-dependent analysis is cheap, sensitive, and safe, as there is no requirement for hazardous probes; therefore, it is commonly performed for detection of the miRNA expression profile (Varkonyi-Gasic et al. 2007). Unlike the SYBR®-based quantification technique, the TaqMan® method utilizes a target-specific fluorescent probe, which allows quick detection and quantification of chosen miRNAs (Fig. 11.2). The disadvantage of this method is use of fluorescently tagged oligonucleotide probes, making it an expensive technique, and this is also applicable to validation of novel miRNA in small experiments (Mestdagh et al. 2008).

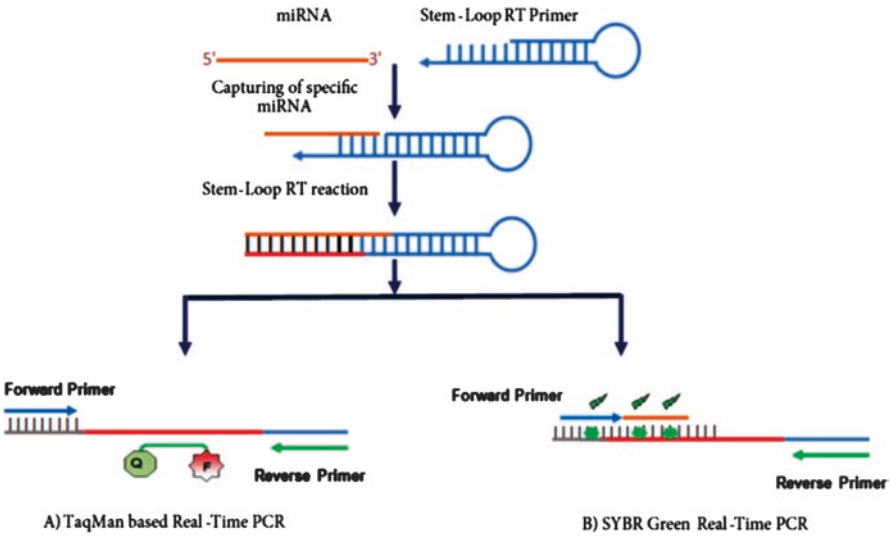


Fig. 11.2 Schematic diagram of a TaqMan® probe and SYBR® Green real-time PCR method for quantification of miRNA (Varkonyi-Gasic et al. 2007)

11.3.3 Northern Blotting

Northern blotting is one of the most common and easiest hybridization-based technologies for miRNA expression profiling at the transcription stage. It includes size-dependent separation of RNA samples by the use of electrophoresis in an agarose gel in denaturing conditions, followed by transfer of RNA to a membrane and recognition of a target sequence through a hybridization probe paired to it. However, this technique has certain negatives, such as its requirements for a time-consuming protocol and for large amounts and high-quality RNA samples; for example, if the samples are even marginally degraded, the excellence of the data and the capability to measure expression are harshly conceded (López-Gomollón 2011). Some modifications were reported by Valoczi et al. (2004), using improved oligonucleotides consuming protected nucleic acid (LNA), which raises the superiority of the method for mature miRNA detection (Valoczi et al. 2004).

11.3.4 In Situ Hybridization

In situ hybridization (ISH) is a useful and adaptable technology, which offers information about the location of mRNA within the tissue, which is a vital stage for analyzing the function, organization, and regulation of genes. The procedure involves precise binding between a nucleic acid probe and complementary sequences of nucleic acid in fixed tissue, followed by imaging the site of the probe. Unlike

northern blot analysis, ISH does not need electrophoretic separation of RNA. There are some key disadvantages associated with the ISH technique, such as the fact that it is time consuming, is tough, and necessitates particular tools for sample preparation and analyzing outcomes of the experiment. Moreover, no direct quantitation of gene expression is possible, in contrast to other techniques. A number of authors have described complete procedures for use of the ISH method in plants (Juarez et al. 2004; Kidner and Timmermans 2006). Kinder and Timmermans (2006) explained the function of miRNAs in *Arabidopsis* and maize by utilizing an miRNA ISH technique. Moreover, Várallyay and Havelda (2011) defined an ISH protocol for miRNA expression analysis in *Arabidopsis thaliana* and *Nicotiana benthamiana* by utilizing extremely sensitive LNA-modified oligonucleotide probes (Várallyay and Havelda 2011).

11.3.5 High-Throughput Sequencing of miRNAs

New high-throughput sequencing (HTS) techniques have been developed as a sensitive, specific, inexpensive, and valuable method for digital gene expression profiling of miRNA. The experimentally derived miRNA sequences are alinged to known miRNA sequences rather than the whole genomic sequence of the target organism (Velasco et al. 2007). This recent technique is more encouraging and is related to qPCR and microarray, as HTS provides a genome-wide method and permits disabling the boundaries of an array-based investigation, which is limited to miRNA molecules delivered by databases. This technology does not need reverse transcription of RNA into cDNA or PCR steps. Moreover, this technique can also be used to determine unique miRNAs. New-generation sequencing (NGS) also permits recognition of species-specific miRNAs (Jones-Rhoades and Bartel 2004; Jones-Rhoades et al. 2006; Voinnet 2009), cells (Judson et al. 2009; Wei et al. 2011), and tissue on a large scale (Chi et al. 2011). Differential expression of miRNAs under a variety of stress conditions, including drought, has also been identified by a number of researchers using NGS methods (Barrera-Figueroa et al. 2011; Wang et al. 2011).

11.4 Summary and Conclusion

A summary of the most recent studies on water stress–mediated miRNA expression and its regulation in plants has been presented in this chapter. This study may facilitate uncovering of the mechanism of action of miRNAs in regulating plant responses to abiotic stress, especially in water deficiency–induced stress. Water stress limits crop production as well as the yield of plants throughout the world. Numerous miRNAs have been identified to be involved in drought stress responses as regulatory molecules. The miRNAs function as both negative and positive regulators of genes against many stresses in plants. Though several drought-regulated miRNAs have

been recognized in a number of plants, the objects of these miRNAs are as yet unidentified. Therefore, we must make it a major task to find out the miRNA targets and their regulatory mechanisms especially in medicinally valuable crops. Further studies on transcription factors and upstream regulatory motifs will also be needed in order to construct a detailed miRNA gene-governing network. Consequently, genetic modification of crops may be possible to make them stress tolerant so that they can withstand adverse conditions.

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Chapter 12
Molecular Markers and Marker-Assisted Selection in Crop Plants

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Abstract Molecular markers have revolutionized the plant biotechnology and genetic studies because of their versatility. These markers include biochemical constituents like secondary metabolites and macromolecules, viz., proteins and deoxyribonucleic acids (DNA). The secondary metabolites are specific to particular plants species that produce them; therefore, the technique has a restricted application. Also, these are influenced by the environmental factors and/or management practices. The molecular markers based on the DNA polymorphism, however, are more suitable and ubiquitous to most of the plant species. These are stable and could not be influenced by environmental factors and/or management practices. Among the other applications of DNA markers, the most promising for plant breeding is marker-assisted selection (MAS). The application requires the markers to be linked with genes of economic significance, cost-effective, and applicable to large number of samples as well as a wide range of crosses in a breeding program. In this chapter, we review the literature about molecular markers, their advantages, disadvantages, and the applications of these markers in marker-assisted selection (MAS) in crop plants.

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In recent years, an increasing availability of genomic information advancement is seen at enhanced pace in plant genetic engineering and breeding. The development of markers for genetic and biochemical analysis has led to the intensification in our understanding of the structure and behavior of various crop genomes and proteomes, expanding our knowledge base for modulation and manipulation of crops for higher yields, better quality of produce, and tolerance to biotic and abiotic stresses. Markers based on genetic makeup are distinguishing features of an individual on the basis of which they can be sorted out from the population. Genetic markers may be divided into classical and DNA-based markers or molecular markers (Collard and Mackill 2008). Classical markers may be morphological, e.g., days to maturity, flower color, seed shape, disease resistance, etc.; cytological, e.g., banding pattern, width, order and position of chromosomes; and biochemical, e.g., allozymes (Howe et al. 2003). Phenotype-based genetic markers have been used by early plant breeders for selection of plants. Mendel had also used phenotype of the plant as genetic marker for selecting individuals in his experiments. Later on theory of genetic linkage in *Drosophila* was established using these markers. The major drawback of both morphological and biochemical markers is that they are restricted in number and are influenced by the environmental factors and plant developmental stages (Pérez-de-Castro et al. 2012).

Molecular marker may be defined as an identifiable piece of DNA that represents variations among the individuals at the genomic level (Vaseeharan et al. 2013; Collard and Mackill 2008). Molecular markers are advantageous over morphological markers as they are stable, distributed throughout the genome, follow Mendelian pattern of inheritance, locus specific, reproducible, and are not influenced by the environment and pleiotropic as well as epistatic effects (Kumar et al. 2010). Molecular markers are useful in a variety of ways to assist plant breeding programs like detection of allelic variations that exist for a gene and are responsible for expression of the traits due to the presence of genetic linkage (Collard and Mackill 2008), marker-assisted procedures for germplasm improvement and varietal development, incorporating multiple genes (gene pyramiding) for resistance to biotic and abiotic stresses into the elite variety (Varshney et al. 2007), germplasm characterization, genetic diagnostics, characterization of transformants, and the study of genome organization as well as phylogenetic relationships (Varshney et al. 2007). The rate and accuracy of selection of individuals in a selection process can be increased using molecular markers (Liu et al. 2013). The molecular markers should have the following characteristics:

1. They should be polymorphic (different form of a trait), so that the chromosome containing mutant gene can be differentiated from the chromosome with normal gene.
2. They should reveal accurate resolution of genetic variations and should be highly reproducible.
3. They must be reliable, multi-allelic, independent, cost-effective, quick, and easy to generate.

4. They should not require prior information regarding the organization of the genome of the individual and must be linked with the target trait which is to be distinguished.

Molecular markers can be classified as follows:

1. First-generation or non-PCR-based markers
2. Second-generation or PCR-based markers
3. New-generation molecular markers
4. Advance markers

12.1 First-Generation or Non-PCR-Based Molecular Markers

12.1.1 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) marker is a first-generation molecular marker. It was first used for analysis of DNA polymorphism among different individuals. It is non-PCR- or hybridization-based technique (Botstein et al. 1980). In RFLP, DNA samples of various plants are isolated and digested with restriction endonucleases. These restricted fragments of DNA are then transferred onto nitrocellulose membrane and hybridized by a labeled DNA probe. These DNA probes are mostly single-locus probes of about 0.5–3.0 kb in length and species specific. They are obtained from cDNA library or genomic library and may be labeled using radioactive isotope or nonradioactive dyes such as digoxigenin or fluorescein (Kumar et al. 2010). After hybridization, variable DNA profile is obtained. These differences in DNA profile appear due to the nucleotide substitutions or DNA rearrangements like insertions, deletions, or single-nucleotide polymorphisms. The RFLP markers are codominant in nature and, thus, able to detect homozygous and heterozygous individuals in F_1 generation. They are locus specific and reproducible. No prior genome sequence information is required and is relatively simple to score due to bulky size variation between fragments. RFLP markers are considered superior because of their high heritability, locus specificity, and distribution throughout the genome. The limitation of these markers, however, is the requirement of expensive radioactive/toxic chemicals, large quantity of high quality genomic DNA, prior genomic information for generation of probes, low level of polymorphism, detection of few loci per assay, and the time consumed (Kiran et al. 2010).

First RFLP linkage map was constructed for tomato and later on it was used for the first time to resolve quantitative traits into discrete Mendelian factors (Bernatzky and Tanksley 1986; Paterson et al. 1988). RFLPs can be used for a variety of purposes like the study of diversity and phylogenetic relationships, gene mapping, finger printing and studies on hybridization, and gene introgression (Kumar et al. 2010).

12.2 Second-Generation or PCR-Based Markers

Several methodologies were developed for the generation of markers after the discovery of polymerase chain reaction (PCR) technology. In PCR-based techniques, there is no need of prior sequence information of the organism's genome for the generation of marker. There are following two types of PCR-based techniques:

(a) Arbitrarily primed PCR-based techniques
(b) Sequence-targeted PCR-based techniques

12.2.1 Arbitrarily Primed PCR-Based Markers

12.2.1.1 Random Amplified Polymorphic DNA (RAPD)

The RAPD technique is based on the differential PCR amplification of genomic DNA (Williams et al. 1990). The variation in DNA profile occurs due to the rearrangements or deletions at or between oligonucleotide primer sites in the genome. In this technique, random decamer oligonucleotide sequences are usually used as primers. It can be applicable across species using universal primers, as prior knowledge of the genome is not necessary. The main disadvantage of this marker is that it is not reproducible across the laboratory due to the low stringency conditions. It is dominant in nature and thus not able to differentiate heterozygous from homozygous individuals. High-density genetic linkage map was developed using RAPD in many plant species like *Bacop*a (Tripathi et al. 2012), ginger (Ashraf et al. 2014), and faba bean (Torress et al. 1993). The method has also been successful in recognizing markers linked to disease-resistant genes in near-isogenic lines (NILs) of tomato (Martin et al. 1991) and common bean (Adam-Blondon et al. 1994). RAPD marker has also been employed successfully in authentication of medicinal plants from its adulterants (Khan et al. 2009; Sultan et al. 2010).

12.2.1.2 Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)

This technique is the modification of RAPD. It was developed by Welsh and McClelland in 1990. AP-PCR involves autoradiography for visualization of the DNA bands, and the DNA fragments are separated using gel electrophoresis. In this technique, a single 10–15 nucleotides long primer is used for amplification of the genomic DNA of the individual. In this method, initial two PCR cycles are carried out at low stringency and then rest of the cycles are carried out at higher stringency by increasing the annealing temperature.

12.2.1.3 DNA Amplification Fingerprinting (DAF)

In DAF technique, very short (i.e., less than ten) GC-rich oligonucleotide sequence is used as primer for amplification of the genomic DNA (Caetano-Anolle's and Bassam 1993), and the polyacrylamide gel electrophoresis along with silver staining is used for analysis of the amplified bands (Sen et al. 1997). Caetano-Anolle et al. (1991) first reported the importance of short oligonucleotides for DNA amplification, producing genotype-specific DNA profiles.

12.2.1.4 Amplified Fragment Length Polymorphism (AFLP)

AFLP marker system was developed by Vos et al. (1995) to resolve the disadvantage of reproducibility linked with RAPD. This technique utilizes the advantage of both the RFLP and the PCR-based technology. In AFLP, genomic DNA is first digested with two restriction enzymes with a combination of rare cutters (*EcoR I or Pst I*) and frequent cutters (*MSe I* or *Taq* I) to cleave the DNA in fragments of varying length (Chial 2008). The generated segments are distinct and noncomplementary to each other. The two restriction enzymes are used to provide better resolution of the bands on the gel. Then adaptor molecules, i.e., primer recognition sequences, are ligated to the sticky ends of the restricted DNA fragments for selective PCR amplification of these fragments using specific primers. The adopter fragments are short double-stranded DNA molecules of about 19–22 base pairs in length containing primer recognition sequences and few bases complementary to the restricted fragments. Number of amplicons per AFLP assay is based on the number of selective nucleotides in the AFLP primer combination, physical complexity and size of genome, the selective nucleotide motif, and the GC content. DNA fingerprints of an individual are generated without any prior knowledge of its source or genome sequence and still these markers, however, are highly reliable and reproducible (Johnson et al. 2007). It gives good resolution and is not sensitive to reaction conditions (Johnson et al. 2007). A large number of polymorphic loci can be analyzed with a single primer combination at a time, on a single gel. It has potential to amplify DNA fragments between 50 and 100 fragments at one time (Mueller and Wolfenbarger 1999). The amplified products are mostly homologous and locus specific with exceptions of polyploid species. AFLP technique, however, requires DNA free from inhibitors and is quite expensive. As most of the AFLP loci are dominant therefore, this technique is unable to detect heterozygous and homozygous individuals.

Most AFLP fragments can be utilized as landmarks in physical and genetic mapping. The technique can be used to distinguish closely related individuals at the subspecies level (Althoff et al. 2007). AFLP technique is also applied for constructing linkage groups in crosses, saturating regions with markers for gene corridor efforts (Yin et al. 1999), and characterization of genetic diversity among cultivars (Mian et al. 2002; Fjellheim and Rognli 2005; Elazreg et al. 2011). Fluorescence-tagged primers are also used in AFLP for high-throughput screening approach.

Huang and Sun (1999) reported that amplified fragments may be detected on denaturing polyacrylamide gels using an automated AFL DNA sequencer with the fragment option.

Witsenboer et al. (1997) introduced modification of AFLP, known as selectively amplified microsatellite polymorphic locus (SAMPL), to identify codominant microsatellite markers using PCR technology in lettuce. In this technique, microsatellite loci are amplified using one AFLP primer in combination with a primer paired to microsatellite sequences. This technique also does not require prior information associated with the cloning and characterization of microsatellite sequences.

12.2.1.5 Sequence-Characterized Amplified Regions (SCAR)

SCAR marker technique was introduced by Michelmore and Martin in 1991 to overcome the limitation of reproducibility of RAPD. SCARs are PCR-based markers that represent genomic DNA fragments at genetically defined loci and amplified by the PCR using specific oligonucleotide primers (McDermott et al. 1994). In this technique the primers, i.e., 15–30 base pairs, are designed from nucleotide sequences that are derived from cloned terminal sequence of the RAPD fragments (Ye et al. 2006). Polymorphism among the individuals is scored on the basis of the presence or absence of amplified bands on the gel. SCAR markers are stable, reproducible, and easy to use. They do not require prior sequence information and can be performed with low amount of sample DNA (Li et al. 2012; Khan et al. 2008).

SCARs are generally dominant, but can be converted into codominant markers by digesting them with restriction enzymes, and the bands can be resolved by either denaturing gel electrophoresis or SSCP (Kiran et al. 2010). The most significant drawbacks of SCAR markers are the time taken for its development and expenses (Polashock and Vorsa 2002). Despite these drawbacks, the technique is helpful in map-based cloning, identification of marker linked with gene of interest (Gao et al. 2005), defining locus specificity (Paran and Michelmore 1993), genetic mapping, comparative mapping (Guo et al. 2003), physical mapping (Chelkowski and Stepień 2001), and homologous studies among the related species. Species-specific SCAR markers have also been developed for several plants, including *Phyllostachys bambusoides* (Das et al. 2005), *E. globulus* ssp *maidenii* (Richero et al. 2013), *Amaranthus* sp. (Ray and Roy 2009), *Jatropha curcas* L. (Mastan et al. 2012), *Hippophae rhamnoides* L. (Korekar et al. 2012), and *Eucalyptus globulus* ssp *globulus, Cuscuta reflexa* (Abdin et al. 2012) and *Phyllanthus emblica* (Dnyaneshwar et al. 2006).

12.2.1.6 Cleaved Amplified Polymorphic Sequences (CAPS)

In CAPS marker technique, PCR-based markers are developed using DNA sequences obtained from mapped RFLP markers without DNA blotting (Komori and Nitta 2005). CAPS are also known as PCR-RFLP (PCR-restriction fragment length polymorphism) markers (Konieczny and Ausubel 1993). The CAPS reveal that the RFLP arises due to single-base change like SNP and insertion/deletion, and

as a result the restriction endonuclease recognition site is modified in amplified fragments (Konieczny and Ausubel 1993; Chelkowski and Stepień 2001). The locus-specific PCR amplicons are digested with one or more restriction enzymes and then separated on agarose or polyacrylamide gels. The primers for generation of CAPS are synthesized based on the prior sequence information of genomic or cDNA sequences or cloned RAPD bands available in NCBI databank. CAPS analysis can be combined with single-strand conformational polymorphism (SSCP), SCAR, AFLP, or RAPD analysis to enhance the possibility of finding DNA polymorphisms. CAPS markers are locus specific and codominant. Hence, they are able to discriminate homozygous plants from the heterozygous ones (Konieczny and Ausubel 1993). CAPS markers are used for molecular identification studies, positional or map-based cloning, and genotyping (Weiland and Yu 2003; Spaniolas et al. 2006).

Michaels and Amasino (1998) proposed a modification of CAPS called derived cleaved amplified polymorphic sequences (dCAPS) technique to overcome the limitation of these markers. In this method, a restriction enzyme recognition site containing SNP is introduced into the PCR product using primer consisting one or more mismatches to template DNA (Neff et al. 1998). Restriction enzyme is used to digest modified PCR product, and the presence or absence of the SNP is observed by the resulting restriction pattern. The method is easy and relatively inexpensive and utilizes the advantage of PCR technology, restriction digestion, and agarose gel analysis. This method is proved to be useful for positional-based cloning of new genes in plants and tracking the known mutations in segregating populations (Haliassos et al. 1989).

12.2.2 Sequence-Specific PCR-Based Markers

Microsatellite or SSR (simple sequence repeat) markers consist of tandem repeats of di-, tri-, tetra-, penta-, and hexanucleotides. Such repeats are prevalent in almost all prokaryotic and eukaryotic genomes (Tautz and Renz 1984; Katti et al. 2001; Toth et al. 2000; Salem et al. 2008). Also named as sequence-tagged microsatellite sites (STMS), and microsatellite or short tandem repeats markers, these markers are hypervariable in nature (Jiang 2013). The variation in the number of these units contained in an SSR mainly occurs due to DNA strand slippage during replication, where the nucleotide repeats allow matching through excision or addition of repeats (Schlotterer and Tautz 1992). The events of DNA strand slippage during replication occur more frequently than the point mutations. During analysis through PCR, microsatellite assays are able to show interindividual differences called polymorphisms of unique loci. The diverse groups of primer sequences are used for PCR amplification including loci-specific unlabeled primer pairs or radiolabeled or fluorolabeled primers. The unlabeled PCR products can be analyzed using techniques of polyacrylamide or agarose gel electrophoresis. The application of fluorescent-labeled primers and use of automated sequencers in genotyping of individuals using SSR analysis procedures have significantly improved the pace of research (Wenz et al.

1998). The assay, however, becomes costly because of the use of fluorescent-labeled primers. Schuelke (2000) has developed a novel technique of PCR analysis using SSR markers, in which three primers are used for the amplification of a specific microsatellite locus. This technique is proved simple and less expensive. SSRs being codominant in nature are highly desirable and, therefore, able to distinguish heterozygotes from homozygotes, highly reproducible, frequent in most eukaryotes as well as prokaryotes, and highly polymorphic even between closely related lines. The use of polyacrylamide gel electrophoresis for separation of the fragments and longer development time of the gel makes it little expensive. It gives information only about single locus per assay which is another significant disadvantage of this marker. The application of SSR markers in germplasm characterization, development of genetic maps, and identification of QTLs has been acknowledged (Hiremath et al. 2012). The studies on locus-specific microsatellite-based markers have been reported in numerous plant species such as in rice (Wu and Tanksley 1993), barley (Saghai Maroof et al. 1994), lettuce (van de Wiel et al. 1999), chickpea (Nayak et al. 2010), jute (Mir et al. 2009; Das et al. 2012), wheat (Mukhtar et al. 2015), Alfalfa (Li et al. 2009) etc.

12.3 New-Generation Molecular Markers

12.3.1 Inter-Simple Sequence Repeat (ISSR)

This technique was reported by Zietkiewicz et al. (1994). ISSRs are 100–3000 long DNA fragments situated between adjacent, oppositely oriented microsatellite regions. Primers are designed to amplify inter-SSR DNA sequences from the core sequences of the microsatellite and few selective nucleotides as anchors into the non-repeat adjacent regions. The generated loci are separated using gel electrophoresis and scored as the presence or absence of the band of particular size.

Single primer amplification reaction (SPAR) technique is related to ISSR in which single primer having only core motif of microsatellite is derived for amplification. The other ISSR-related technique is directed amplification of minisatellite region DNA (DAMD), which uses a single primer containing only the core motif of a minisatellite.

12.3.2 Single-Nucleotide Polymorphism (SNPs)

SNPs are the most abundant sources of polymorphism. They represent the single base changes at specific locations between the individual genomes (Duran et al. 2009). The changes arise due to the transversions (C/G, A/T, C/A, or T/G), transitions (C/T or G/A), and small insertions/deletions. SNPs are generally biallelic at a particular site, but they may also be tri- or tetraallelic (Doveri et al. 2008). They are

codominant and evenly distributed in higher frequencies throughout the genomes of most plant species ((Vignal et al. 2002; Allen et al. 2011; Yan et al. 2009). SNPs are useful in developing high-density linkage map, genome-wide association mapping, marker-assisted selection, and genomic selection studies (Varshney 2010). They are stable and do not change generation after generation and, therefore, useful in understanding of genome evolution (Syvanen 2001). They may occur in coding as well as in noncoding regions. Their occurrence and distribution varies among species. SNPs can be widely used in a variety of purposes like cultivar identification and genetic mapping. Single-nucleotide polymorphism (SNP) markers put forward the assurance of high map resolution and throughput as well as lower cost and error rate compared to the SSR markers (Jones et al. 2007). Hiremath et al. (2012) developed second-generation genetic map with an average inter-marker distance of 0.59 cM in chickpea using SNP marker. Jones et al. (2009) constructed genetic linkage map in mapping population generated from the cross B73×Mo17 of maize utilizing SNP marker.

12.4 Advance Marker Techniques

The technical improvement and genome-based innovation have led to the enhancement of molecular marker techniques. These highly developed molecular marker methods employ the valuable features of various basic methods as well as incorporation of modifications in the methodology to increase the specificity, sensitivity, and resolution to detect polymorphism at the genetic level among the individuals.

12.4.1 Organelle Microsatellite-Based Markers

The traits governed by the cytoplasmic genes follow uniparental mode of inheritance and, thus, show different patterns of genetic differentiation compared to that of nuclear alleles (Provan et al. 1999a, b). The chloroplast and mitochondrial genomes are useful in understanding of genetic structure, phylogenetic relations, differentiation, and evolution of the plant population. The markers based on chloroplast and mitochondrial microsatellites are unique and, therefore, have also been developed.

12.4.1.1 Chloroplast Microsatellites

Chloroplast microsatellites are ubiquitous and show polymorphism in chloroplast DNA among the individuals. They comprised of several short mononucleotides segments like (dA)n and (dT)n (Powell et al. 1995). Markers, based on chloroplast genome, reveal variations among or between taxa that could not be exhibited by

DNA markers, since interbreeding and genetic exchange have hidden the relics of past demographic patterns (Wolfe et al. 1987). Chloroplast microsatellite-based markers have been used for various purposes like study of patterns of cytoplasmic variation in a wide range of plant species (Provan et al. 2001), mating systems, gene flow via both pollen and seeds and uniparental lineage, recognition of hybridization and introgression (Bucci et al. 1998), genetic diversity analyses (Clark et al. 2000; Yang et al. 2011), and phylogenetic relationships among the taxa and species (Shaw et al. 2005; Awasthi et al. 2012). The main disadvantage of this technique, however, is the need of sequence data for primer construction.

Primers flanking chloroplast microsatellites can only be generated from fully or partially sequenced chloroplast genomes. The polymorphic PCR fragments can be generated from the species of origin and their close relatives. Weising and Gardner (1999) have used a set of consensus chloroplast microsatellite primers (ccmp1 and ccmp10) to design universal primers for amplification of cpSSR regions in the chloroplast genome of dicotyledonous angiosperms. The primer pairs developed from A or T mononucleotide repeats (n = 10) of tobacco chloroplast genome were also functional as genetic markers in different species belonging to the families, Actinidiaceae, Brassicaceae, and Solanaceae (Chung and Staub 2003). Provan et al. (2004) have developed universal primers for amplification of chloroplast microsatellites in grasses.

12.4.1.2 Mitochondrial Microsatellites

In plants, mitochondrial genome is not generally useful in phylogenetic analysis due to a high rate of sequence reorganization (Sederoff et al. 1981). The mitochondrial haplotype diversity arises due to sequence rearrangement and is, however, proved to be useful in population differentiation of pine and fir taxa (Soranzo et al. 1999; Sperisen et al. 2001). Kumar et al. (2007) reported that the mitochondrial microsatellites can be used for trait-based segregation in population.

12.4.2 Randomly Amplified Microsatellite Polymorphism (RAMP)

Randomly amplified microsatellite polymorphism (RAMP) technique was developed by Wu et al. (1994). It utilizes the advantages of both RAPD and microsatellite-based markers. In this technique, genomic DNA is first amplified by using radiolabeled primer containing 50 anchor and 30 repeats in the presence or absence of arbitrary (RAPD) primers. The resulting products are then separated on denaturing polyacrylamide gels, and as the repeat primer is labeled, the amplified products derived from the anchored primer are only detected. RAMP has been employed successfully in genetic diversity studies of the cultivars of barley (Wu et al. 1994;

Sanchez de la Hoz et al. 1996), peach (Cheng et al. 2001), tomato, and kiwi fruit (Shahid et al. 2012).

12.4.3 Sequence-Related Amplified Polymorphism (SRAP)

SRAP technique is utilized to amplify open reading frames (ORFs) (Li and Quiros 2001). It uses pairs of arbitrary primers of about 17–21 long nucleotide sequences containing AT- or GC-rich core sequences. In this technique, intragenic segments are amplified to determine polymorphism. The primers used in this technique are consisted of core sequences (13–14 bases long), where first 10 or 11 bases starting at the 5′ end are the sequences of no specific constitution (filler sequences) that are followed by the sequence, CCGG in the forward primer and AATT in the reverse primer. The core sequences are that followed by three selective nucleotides at the 3′ end. The filler sequences of the forward and reverse primers must be different from each other. The amplified products are separated by agarose or denaturing polyacrylamide gel electrophoresis (Gulsen et al. 2007; Mishra et al. 2011; Ferriol et al. 2003) and visualized by autoradiography. SRAP polymorphism occurs due to the differences in fragments sizes caused by insertions and deletions.

The SRAP technique is a simple, reliable, cost-effective, multilocus, and codominant in nature. It targets open reading frames (ORFs) in the genome of plant species. SRAP marker system has been applied for a variety of purposes in different crops, including map construction (Xie et al. 2011), gene tagging (Devran et al. 2011; Zhang et al. 2011), genetic diversity studies (Gulsen et al. 2006; Aneja 2010), and hybrid identification (Mishra et al. 2011).

12.4.4 Target Region Amplification Polymorphism (TRAP)

TRAP technique is a rapid and efficient PCR-based strategy (Hu and Vick 2003), which uses two primers, about 18 nucleotides long, to generate markers. In this method, polymorphic markers are generated around the targeted candidate gene sequences using bioinformatics tools and expressed sequence tag (EST) database information. The first primer is called fixed primer and is designed from the targeted EST sequence, whereas the second primer is an arbitrary primer containing AT- or GC-rich core and anneal with an intron or exon. TRAP marker is helpful in genotyping of germplasm and generation of markers associated with particular traits in crop plants (Hu et al. 2005). The technique has been efficiently employed in fingerprinting of lettuce cultivars (Hu et al. 2005) to determine genetic diversity in sugarcane (Alwala et al. 2006) and QTL mapping in intervarietal recombinant inbred population of wheat (Liu et al. 2005).

12.4.5 Single-Strand Conformation Polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) is a rapid, easy, and powerful tool for the detection of various types of mutations like substitutions, insertions, and deletions (Fukuoka et al. 1994; Hayashi 1994) and typing of DNA polymorphism in amplified PCR product (Orita et al. 1989). It can determine heterozygosity of DNA and can even detect a few nucleotides (Shahid et al. 2012). In SSCP technique, the two DNA strands from the same PCR products often run separately on SSCP gels and, thus, helpful in scoring polymorphism and reveal internal sequence polymorphism in some PCR products from homologous locations in the two parental genomes (Hayashi 1992). SSCP analysis can also reveal DNA polymorphisms and mutations at multiple places in DNA fragments. This technique is similar to RFLPs in the sense that it can also reveal the allelic variations of genetic traits. To rectify the limitation of reannealing of complex banding pattern, an asymmetric PCR-SSCP was developed. In this method, denaturation step was eliminated. A large-sized sample can be loaded for gel electrophoresis to increase the high-throughput detection of DNA polymorphism in plants (Li et al. 2005). Fluorescence-based PCR-SSCP (F-SSCP) was developed to amplify the target sequence using fluorescent primers (Makino et al. 1992).

12.4.6 Transposable Element-Based Molecular Markers

Transposable genetic elements are the fragments of DNA that can change their positions within the genome (either within a chromosome or between the chromosomes of an organism) by virtue of transposase enzyme and also between the genomes through carrier organisms. Such elements were first discovered in maize by Barbara McClintock in 1948. Transposable elements possess conserved sequences, are thought to have ubiquitous distribution, have abundant copy number, and have high heterogeneity that justifies their use for development of transposable element-based molecular markers (Kalendar et al. 1999; Kalendar et al. 2011; Kim et al. 2011).

Retrotransposon-based markers use a pair of primers, where one primer corresponds to the retrotransposon and the other is complementary to a section of the neighboring genome. Retrotransposon-based molecular markers can be classified as follows.

12.4.6.1 Inter-Retrotransposon-Amplified Polymorphism (IRAP) and Retrotransposon-Microsatellite Polymorphism (REMAP)

The IRAP and REMAP systems were developed by Kalendar et al. (1999) to determine the polymorphism using BARE-I retrotransposon existing in barley genome. In IRAP, primers are designed from the LTR regions, and they amplify the genomic

DNA that lies between the two retrotransposon insertion sites. In REMAP, genomic DNA is amplified by using one outward facing LTR primer and the other primer from a microsatellite sequence. Primers are designed to the microsatellites and are anchored to the microsatellite's 3′ terminus by the addition of a single selective base at the 3′ end. The polymorphism is detected on the basis of the presence or absence of the bands.

IRAP marker system has been used in genome classification of banana cultivars (Nair et al. 2005). Branco et al. (2007) used both IRAP and REMAP markers to detect similarity between 51 rice cultivars.

12.4.6.2 Sequence-Specific Amplification Polymorphism (S-SAP)

This technique is a modification of the amplified fragment length polymorphism (AFLP) and was first used to identify the location of BARE-1 retrotransposons in the barley genome (Waugh et al. 1997). In this method, genomic DNA was first digested with two different restriction enzymes to generate the template DNA for PCR amplification. The primer pair is designed from the LTR region of the retrotransposon and the selective bases of the adaptor molecules to amplify the genomic DNA. S-SAP marker system is highly polymorphic and codominant as compared to the AFLP marker (Lou and Chen 2007). It uses restriction enzymes and, therefore, the cost of the method is high. S-SAP has been exploited efficiently in different plant species like pea, Medicago, lettuce, wheat, apple, and the cashew for genetic diversity analysis and in the construction of linkage maps (Pearce et al. 2000; Porceddu et al. 2002; Queen et al. 2004; Syed et al. 2006; Zhao et al. 2010). Another technique similar to the S-SAP in which DNA transposon is applied instead of retrotransposon is known as transposon display (TD). This marker has been developed in rice with high polymorphism and reproducibility (Shcherban et al. 2000; Kwon et al. 2005).

12.4.6.3 Retrotransposon-Based Insertion Polymorphism (RBIP)

Flavell et al. (1998) developed the technique of RBIP using the PDR1 retrotransposon in pea. The technique uses the sequence information from the 5′ and 3′ flanking regions of the transposon. A primer specific to the transposon along with a primer designed to anneal to the flanking region is used to generate a product from template DNA containing the insertion. On the other hand, if the insertion is absent, primers specific to both flanking regions also amplify a product. The DNA polymorphism is detected by using agarose gel electrophoresis or by hybridization with a reference PCR fragment. The identification of polymorphism through hybridization is more informative for automated, high-throughput analysis. Hybridization-based assays are however discouraged in view of their high cost and technical complications.

12.4.6.4 Transposable Display (TD)

This technique allows simultaneous detection of many transposable elements (TEs) from a high copy number lines of a given plant species. Being modification of AFLP technique here, primers are designed from the anchored region of the restriction sites (i.e., BfaI or MseI) and a transposable element rather than the two restriction sites (van den Broeck et al. 1998). In this technique individual transposons are identified by a ligation-mediated PCR. This ligation-mediated PCR starts from within the transposon and amplifies portion of the flanking sequence up to a specific restriction site. The resulting PCR products are analyzed in a high-resolution polyacrylamide gel system. In petunia, Van den Broeck et al. (1998) detected the copy number of the dTph1 transposon (TIRs) family and related insertion events. This technique can also be used to detect insertions that can be correlated with a particular phenotype. In maize, Casa et al. (2000) could exploit TD technique to develop a new class of molecular marker (Hbr transposon family) from the miniature inverted-repeat transposable elements (MITEs) for detecting polymorphism.

12.4.6.5 Inter-MITE polymorphism (IMP)

In this technique, miniature inverted-repeat transposable elements (MITEs) like transposons are used in place of retrotransposons. MITEs are abundant in plant genomes in the form of short, nonautonomous DNA elements. They have advantage of detecting high copy number and intra-family homogeneity in size and sequence. On the basis of structural and sequence characteristics, MITEs are divided into two major groups: tourist-like and stowaway-like. The IMP technique was primarily employed to identify two groups of MITEs in barley, one from stowaway family and the other of barfly family (Chang et al. 2001).

12.4.6.6 RNA-Based Molecular Markers

cDNA-AFLP and RAP-PCR are the PCR-based molecular marker techniques which are used to amplify cDNAs for differential RNA study.

(i) ***RNA fingerprinting by arbitrarily primed PCR (RAP-PCR)***
RAP-PCR technique was given by Welsh et al. (1992). In this technique, arbitrarily selected primers are used to amplify cDNA population for fingerprinting of RNA populations. In this method variable PCR products are obtained for RNAs isolated from different tissues of the same individual and from same tissue of the different individuals. It is helpful in mapping of genes due to sequence polymorphism, and the tissue-specific differences revealed are valuable in understanding the differential gene expression.

(ii) *cDNA-AFLP*

This technique is utilized to demonstrate differentially expressed genes (Bachem et al. 1996). In this method, cDNAs are digested by two restriction enzymes and then ligated by oligonucleotide adapter molecules. Primers complementary to the adapter sequences with additional selective nucleotides at the 3′ end (Bachem et al. 1998) are used for PCR amplification. The cDNA-AFLP technique is more reproducible than RAP-PCR (Liang and Pardee 1992). It can distinguish between highly homologous genes from individual gene families and does not require prior sequence information. It is useful in identification of the genes involved in various novel processes (Yaa et al. 2007). cDNA-AFLP technique is most useful in identification of stress-regulated genes in various plant species (Mao et al. 2004).

12.5 Marker-Assisted Selection (MAS) in Crop Plants

Marker-assisted selection is a genotype-based selection process in which plants with desirable features are chosen from the segregating generations with the help of molecular markers allied strictly with the target traits (Choudhary et al. 2008; Barrett et al. 2001). The discovery of molecular markers dispersed all over the genome and their correlation with desired traits made marker-assisted selection a reality. Isozymes had been used for the first time in the 1980s as molecular markers for introgression of qualitative traits from exotic germplasm to adapted cultivar (Xu and Crouch 2008). It is most suitable for the selection of those traits, which are complex in nature, and the traits that require specific environmental condition for their expression. It is also used in tracking the recessive genes in backcross progenies and for the incorporation of multiple genes into a single variety (Collard et al. 2005; Araujo et al. 2010).

The advantages of MAS include time saving due to selection of desirable plants at seedling stage, elimination of linkage drag during backcross programs, useful in selection of traits having low heritability, elimination of unreliable phenotypic evaluation related to field trial due to environmental effects, and incorporating many genes simultaneously (Siangliw et al. 2007). The pre-requisites for MAS are (1) availability of well-saturated genetic linkage map, (2) tight linkage between the gene of interest and adjacent marker, (3) adequate recombination between the marker and rest of the genome, and (4) the ability to screen a large number of individuals in less time and cost-effective manner (Babu et al. 2004). Its success depends on the relationship between the marker and the gene of interest. The following three kinds of relationship are found between the maker and the gene in a population:

1. The most favorable condition for marker-assisted selection is when the candidate marker gene is located within the gene of interest. To find this kind of relationships is, however, difficult. This condition may be referred as gene-assisted selection (GAS).

2. The marker is in linkage disequilibrium (LD) with the gene of interest throughout the population. LD is the propensity of a group of alleles to be inherited together. This condition may be prevailed, when lines or genotypes have been crossed in recent generation or marker and genes of interest are physically very close to each other. When plants are selected using these markers, then the technique is called LD-MAS.
3. The situation in which marker is not in linkage disequilibrium (LD) with the target gene throughout the whole population. This situation is the toughest situation in MAS. Selection by means of these markers can be called LE-MAS.

12.5.1 Marker-Assisted Evaluation of Breeding Material

In breeding program, DNA marker may be utilized in a variety of purposes like cultivar identification, assessment of genetic diversity, selection of parent for hybridization, and confirmation of hybrids. Conventionally, above tasks have been done based on visual selection, and data are analyzed using phenotypic characteristics of the plants.

12.5.1.1 Cultivar Identification and Assessment of Genetic Purity

In practice, seed of different varieties of the same crops are generally mixed during the handling of large number of seed samples during breeding programs. Molecular markers may be utilized to identify individual plants from the mixture of the seeds of different varieties. Maintenance of genetic purity of the parental lines is necessary to exploit maximum heterosis during hybrid seed production. In this regard, molecular markers have been successfully exploited to confirm the purity, and it is simpler than the conventional grow out test in which plant is grown up to maturity and assessed on the basis of morphological and floral characteristics.

12.5.1.2 Assessment of Genetic Diversity and Parental Selection

Any crop improvement program relies on the presence of genetic variability for a target trait in a population and selection of the desirable individuals. The genetic variability may be natural or may be created through different breeding methods like hybridization, mutation, soma clonal variation, genetic engineering technique, etc. Various studies have been reported by several workers regarding the assessment of genetic diversity within breeding population using molecular markers.

12.5.1.3 Study of Heterosis

Development of superior inbred line is one of the most important steps in hybrid development program. The development of inbred lines through conventional methods for utilization in hybrid crop production, however, is laborious and expensive. Inbred development using marker-assisted selection makes hybrid development cost-effective. DNA-based markers have also been used to characterize heterotic groups in hybrid crop production to exploit maximum heterosis in different crops like maize, sorghum, etc. (Lee et al. 1989; Reif et al. 2003). The exact level of heterosis could not be predicted till date using DNA marker data. DNA marker data may, however, be useful in combination with phenotypic data for selection of heterotic hybrids (Jordan et al. 2003).

12.5.1.4 Identification of Genomic Regions Under Selection

Breeders used information regarding the frequency of specific alleles or haplotypes in designing appropriate breeding strategy for crop improvement programs. Therefore, identification of shifts in allele frequency within the genome is important. Identification of genomic regions under selection is also important in QTL mapping (Jordan et al. 2004; Bernardo 2008). Finally, the identified genomic regions can be used for the expansion of new varieties with specific allele combinations using different marker-aided selection schemes.

12.5.2 Marker-Assisted Backcrossing

Backcross was widely used by early breeders for introduction of one or few desirable genes from donor sources to an adapted or elite cultivar (Panihrahi et al. 2013). The improvement of cultivar using conventional breeding is time consuming and laborious. It also inevitably leads to the integration of undesirable chromosome segment of donor parent in the genome of the given cultivar in addition to the desired gene. As a result, it looks like the adapted variety in appearance, but fails to perform exactly like the popular cultivar, and thus limit its use by the farmers for commercial cultivation. Marker-assisted backcross breeding is helpful to overcome these shortcomings by allowing breeders to select plant in early developmental stages with the desired trait and facilitating the removal of undesirable chromosomal segments of the donor from intermediate backcrosses (Babu et al. 2004). Consequently, marker-assisted backcross breeding involves two to three backcross generation and five generations of selfing to develop improved variety similar to popular variety in all respects, except that it possesses the transferred desirable gene (Suh et al. 2011; Ribaut et al. 2002). The efficacy of marker-assisted backcross breeding depends on availability of closely linked DNA marker for the target gene, population size, and

number of backcross (BC) generations along with the location and number of markers for background selection (Frisch and Melchinger 2005).

Backcross breeding using molecular marker involves three steps:

1. Tracking of the presence of target alleles or genes. This is called foreground selection (Hospital and Charcosset 1997) and is beneficial for selection of target traits that require laborious and time-consuming phenotypic screening procedure. Using marker-assisted selection approach, plants can be selected in early developmental stage for those traits which express in later stages. Apart from this, both dominant and recessive alleles of the target gene can also be selected and incorporated by this approach, which is time consuming and difficult to do using conventional approach (Panihrahi et al. 2013).
2. Selection of reconstituted genome of recurrent parent is also known as background selection. This may refer as use of tightly linked flanking markers for recombinant selection and unlinked markers for selection of recurrent parent genotype. Background selection is very important since it accelerates the recovery of recurrent genotype and only BC_4 or even BC_2 generation is required to achieve this goal and thus save two to four backcross generations.
3. Identification of those backcross (BC) progenies having target locus and recombination events between the target loci and linked and/or flanked markers (Holland 2004). This third step is also called recombinant selection. Its objective is to minimize the linkage drag. Using marker-assisted backcrossing, linkage drag can be minimized only by two BC generations, while it is difficult through conventional breeding and the large chromosome segment of the donor DNA remains intact in recipient chromosome of BC generations.

12.5.3 Early-Generation Marker-Assisted Selection

Initially plant breeders deal with a large number of populations, and it is not easy to handle such populations in subsequent generations. Besides, it is also difficult to select plants in early generation for the traits governed by polygenes. Hence, the selection of plants with desirable gene combinations in early generation using molecular markers is required and called early-generation marker-assisted selection. Early-generation marker-assisted selection is useful in eliminating plants with undesirable gene combinations in early generation and, thus, reduces the population size to be handled in subsequent generations. The efficiency of MAS in early generations depends on the probability of recombination between the marker and QTL. The major disadvantage of MAS in early-generation selection is the high cost of genotyping of large number of plants during selection process.

Ribaut and Betran (1999) introduced single large-scale MAS (SLS–MAS) of early-generation marker-assisted selection in which MAS is performed on F_2 or F_3 populations derived from superior parents to select up to three QTLs in a single MAS step using flanking markers. The QTLs targeted for selection should be stable

across the environments and responsible for the largest proportion of phenotypic variance. Ribaut and Betran (1999) suggested that the population size used for early-generation marker-assisted selection should be large enough, so that the genetic drift to occur at nontarget loci may be avoided. Instead of F_2 population, F_3 populations can also be used to minimize this problem. They also proposed that, theoretically, linkage drag could be minimized by using additional flanking markers surrounding the target QTLs, alike MAB.

In self-pollinated crops, it is possible to fix specific alleles in their homozygous state in F_2 generation using DNA codominant marker. In contrary to this, conventional methods require F_5 or F_6 generations to fix alleles in homozygous state.

The other alternative of early-generation marker-assisted selection is combined MAS. It was proposed by Moreau et al. (1998). This approach is useful in the condition when complementary QTLs defining a trait remain anonymous or when a large number of QTLs need to be manipulated. This scheme is advantageous over phenotypic selection or MAS alone in order to maximize genetic gain. Simulation studies have indicated that this approach has an edge over phenotypic screening alone, especially when studying large population sizes where trait heritability is less. Bohn et al. (2001) recommended that the relative efficiency of MAS was increased, when it was used in combination with phenotypic selection, while studying on insect resistance in tropical maize. Marker-assisted selection is advantageous over phenotypic selection for those traits where marker-based genotyping is cheaper than phenotypic screening-like quality traits (Han et al. 1997). The process is often referred to as "tandem selection" (Han et al. 1997) or "stepwise selection" (Langridge and Chalmers 2005).

12.5.4 Marker-Assisted Gene Pyramiding

Marker-assisted gene pyramiding is the process of combining more than one gene from multiple parents into a single genotype using marker-assisted selection (Lan and Chao 2011; Varshney et al. 2013). Gene pyramiding using conventional method alone is not feasible due to the presence of linkage with undesirable traits. Further, it is not possible to remove undesirable linkage with repeated backcrossing (Tanksley et al. 1989).

The objectives of gene pyramiding are to enhance trait expression by combining two or more complementary genes, rectifying weakness of the adapted variety by incorporating genes from other sources, increasing the durability of disease and pest resistance, and broadening the genetic base of related crops. Gene pyramiding can be applied in the following cases (Ashikari and Matsuoka 2006; Shanti et al. 2010):

1. It is applied for those attributes which are monogenic, but difficult to measure phenotypically and those which require specific environmental conditions for their expression.

2. It is also used for improvement of quantitative traits, if major genes affecting the traits are identified. Genes with very similar phenotypic effect, which are impossible or difficult to combine in a single genotype using phenotypic selection, can be combined through MAS.
3. It is applicable in identification of recessive genes, when its effect is being masked by dominant genes.

Gene pyramiding involves two steps. In the first step, all the desirable genes are accumulated into single genotypes, i.e., root genotype called pedigree step, and in the second step desirable genotype is derived with all target alleles from the single genotype called fixation step. Pedigree step is represented by a binary tree, and each node of a tree has intermediate genotype having two parents. The selection of intermediate genotypes is done among offsprings of the crosses which bear all the target parental genes. Within the subset of an intermediate genotype, the desirable alleles are in coupling phase while favorable alleles between subsets are in repulsion phase. The gene fixation step in gene pyramiding program can be achieved using the following methods:

1. The ideal genotype with the desired genes can be obtained by generating diploid population from root genotype. In this method, the target genotype can be derived just after the root genotype is developed.
2. The other procedure is selfing of the root genotype directly to obtain the target genotype. However, this method takes more time as compared to the previous one.
3. In another method, the genotype possessing all the possible desirable alleles is derived by crossing the root genotype with a parent having none of the favorable alleles. The ideal genotype can be obtained within two generations after the root genotype.

The crossing of root genotypes with one of the founding parents instead of a blank parent is also a method to develop ideal genotype. This method is called as marker-assisted backcross gene pyramiding. The marker-assisted backcross gene pyramiding method is most efficient and acceptable method for the gene pyramiding.

Marker-assisted backcross gene pyramiding can be successfully performed using the following three approaches:

1. In the first approach, the recurrent parent is crossed with the donor parent to produce F_1 hybrid. The F_1 hybrid is then backcrossed up to BC_3 generation to recover the recurrent parent. This improved recurrent parent is then again crossed with the other donor parent to accumulate multiple genes. This approach is time taking and incorporate one gene at a time. This approach is most effective, but less acceptable.
2. In the second approach, recurrent parent is crossed with a number of donor parents to derive F_1 hybrid. The F_1 hybrid is then intercrossed to get improved F_1 population. The improved F_1 hybrid is then backcrossed with the recurrent parent to produce improved recurrent parent. When different donor parents are used in this method, there is a chance to lose pyramided genes.

Table 12.1 Marker-assisted gene pyramiding in some important crops

Crop	Agronomic traits	Pyramided gene (s)	Reference(s)
Rice	Bacterial leaf blight resistance	*Xa7* and *Xa21*	Zhang et al. (2006)
		Xa5, *Xa13*, and *Xa21*	Kottapalli et al. (2010) and Sundaram et al. (2010)
		Xa3 and *Xa26*	Li et al. (2012)
		Xa4, *Xa5*, *Xa13*, and *Xa21*	Bharathkumar et al. (2008) and Shanti et al. (2010)
		Xa4, *Xa21*, and *Xa27*	Luo et al. (2012)
	Aroma and pest resistance	*Fgr and Cry 1Ab*	Kiani et al. (2012)
	Striped stem borer resistance, bacterial leaf blight resistance and herbicide resistance	*cry1Ab*, *Xa21*, and *bar*	Wei et al. (2008)
	Bacterial blight resistance and aroma	*Xa5*, *Xa13*, and *Xa21* and *fgr* genes	Salgotra et al. (2012)
	Spikelet number per panicle and 1000 grain weight	Eight QTLs	Zong et al. (2012)
	Blast resistance	*Pi1*, *Pi2*, and *D12*	Jiang et al. (2012)
	Bacterial blight, yellow stem borer, and sheath blight	*Xa21*, *cry1Ab*, *RC7 Chitinase*, and *cry1Ac*	Datta et al. (2002)
	Brown plant hopper	*Bph1* and *Bph2*	Sharma et al. (2004)
	Green rice leafhopper	Grh1, Grh2, Grh3, Grh4, Grh5, and Grh6 and QTL qGRH4	Fujita et al. (2010)
	Gall midge resistance	*Gm1* and *Gm4*	Kumaravadivel et al. (2006)
		Gm2 and Gm6(t)	Katiyar et al. (2001)
Cotton	Fiber strength and pest resistance	QTLs and *Cry1A*	Guo et al. (2005)
Wheat	Powdery mildew	*Pm2* and *Pm4a*	Liu et al. (2000)
	Leaf rust resistance	2 genes	Singh et al. (2004) and Bhawar et al. (2011)
	Cyst nematode resistance	*CreX* and *CreY*	Barloy et al. (2007)
	Powdery mildew resistance	3 genes	Gao et al. (2005)
	FHB resistance	3 QTLs	Miedaner et al. (2006)
	FHB resistance and DON content	3 QTLs	Wilde et al. (2007)
	Yellow rust	*Yr5* and *Yr15*	Santra et al. (2006)
	Leaf rust resistance	*Lr1*, *Lr9*, *Lr24*, *Lr47*	Nocente et al. (2007)
Barley	Yellow mosaic virus resistance	*rym1* and *rym5*	Okada et al. (2004)
		Rym4, *rym9*, and *rym11*	Werner et al. (2005)

(continued)

Table 12.1 (continued)

Crop	Agronomic traits	Pyramided gene (s)	Reference(s)
Soybean	Soybean mosaic virus resistance	*Rsv1*, *Rsv3*, and *Rsv4*	Shi et al. (2009) and Saghai Maroof et al. (2008)
	Asian soybean rust	*Rpp2, Rpp4, and Rpp5*	Yamanaka et al. (2013)
Pearl millet	Downy mildew resistance	P7–3 and P7–4	Hash et al. (2006)
Chickpea	Resistance to pod borer	*Cry1Ab* and *Cry1Ac*	Mehrotra et al. (2011)

3. In another alternative approach, the gene pyramiding can be achieved by simultaneous crossing with many donor parents and then backcrossed them up to BC_3 generations. The backcross populations having individual gene are then intercrossed with each other to produce the pyramided lines. This approach is most efficient and acceptable procedure, because it takes less time for fixation of the genes.

Developing elite breeding lines and varieties using marker-assisted gene pyramiding to combine desirable traits from multiple parental lines has been successful in number of important crop plants (Table 12.1).

12.5.5 Marker-Assisted Recurrent Selection (MARS)

Marker-assisted recurrent selection was proposed by Hospital et al. (2000). It is a marker-based population improvement program in which desirable genotypes are isolated from the breeding population derived from the crosses that contain genomic regions exhibiting smaller QTLs (Varshney et al. 2011). The objective of MARS is to increase the frequency of favorable QTLs in the population through cycles of MAS for multiple QTLs and intermating of the selected individuals in the population in a recurrent selection scheme (Peleman and van der Voort 2003; Ribaut and Ragot 2007). The base population in this scheme is generally F_2 that is derived from the single crosses or backcrosses. Three-way crosses and double crosses may also be used.

In MARS, initially new QTLs are identified from the breeding population originated from the good ×good variety crosses. After that, the lines containing desirable alleles for maximum QTLs are crossed to accumulate superior alleles in one genetic background. The best lines are selected on the basis of phenotype from the recombined lines, and the best one is tested at multi-location field testing to release them as variety. MARS is helpful in accumulation of more number of minor as well as major QTLs in a single genetic background, which is not possible using marker-assisted backcross breeding because it requires large number of population that is difficult to handle (Varshney et al. 2011). The main drawback of this procedure however, is that it requires extra number of generations for cyclical marker based selection. MARS have been used in crops like wheat

(Charmet et al. 2001; Kuchel et al. 2007), sorghum (Abdallah et al. 2009) and chickpea (Varshney et al. 2013).

12.5.6 Genomic Selection (GS) or Genome-Wide Selection (GWS)

This approach is utilized to identify superior lines with higher breeding value in a breeding program using genome-wide marker data. The starting population in genomic selection is training population and candidate population. Training population consist of breeding lines which were/or are in use in breeding program, and overall performance (like yield and component traits) of those lines across the environment are available. Candidate population is that population which is being currently used by breeders and may be derived from the parental lines present in the training population.

In genomic selection scheme, initially genotyping is carried out of all the individuals of the training population, using a large number of markers by considering linkage disequilibrium in the collected breeding germplasm. Statistical models are developed to estimate genomic estimated breeding value (GEBVs) of the lines. Similarly, genotypic marker data is also generated from the candidate population, and GEBVs of the progenies of the candidate population are estimated using statistical models. The lines having higher GEBVs are selected for attempting the next crosses. The phenotypic data of the candidate population, if available, can be used together with the genotypic data for strengthening the model developed based on the training population. The progenies from the selected lines (those having higher GEBVs) of the candidate population are genotyped with the same set of markers. Superior progenies can be selected on the basis of GEBVs calculated based on these marker data using the model. These selected progenies can be used for next cycle of crossing or can be selfed for field evaluation in targeted environments and could be progressed toward multi-location field trials. Genomic selection takes less time. It is cost-effective, because it reduces the frequency of extensive phenotyping and avoids the need of QTL mapping. The reliable phenotype can be developed in less time by minimizing the length of selection cycle. The use of appropriate statistical model for estimating the GEBVs is very important in the success of GS. Bernardo and Yu (2007) suggested that best linear unbiased prediction (BLUP) or Bayesian methods are highly effective for estimating GEBVs. Weighed Bayesian shrinkage regression (wBSR) model may also be a choice of model because it reduces the computational difficulty in MCMC-based Bayesian methods (Takeshi and Hiroyoshi 2010). Various workers are engaged in developing statistical models and/or applying genomic selection in breeding of some crops (Crossa et al. 2010; Karkkainen and Sillanpaa 2012; Jannink et al. 2010; Piepho 2009). Varshney et al. (2013) suggested that it may also be utilized in near future in at least chickpea and ground nut.

12.6 Conclusion

With the better understanding of molecular processes in plants and advancement of biochemical and molecular techniques, plant breeding has remarkably contributed towards crop improvement. Recently developed high-throughput genotyping techniques allow breeders to used marker-aided screening of many genotypes and help them to shift from traditional breeding to marker-aided selection (MAS). These markers have advantages like even distribution throughout the genome, high polymorphism, frequent occurrence, codominant inheritance, and selectively neutral behavior. The technique is easy, fast, economically viable, and reproducible. It has high throughput and transferability between laboratories, populations, and/or species. To realize the potential of MAS, specific strategies need to be tailored with respect to specific markers, crops, traits, and available budget. Further, understanding of molecular biology of disease resistance, defense responses, and the signal transduction leading to activation of defense responses should allow MAS to become more widely applicable for crop breeding programs.

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Chapter 13
Plant-Based Edible Vaccines: Issues and Advantages

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Abstract Transgenic plant technology is proving to be making wonders in its applicability. Apart from its use in improving the crop yield, quality, etc., it is now being exploited to cure human and animal diseases. Foreign proteins from human and animal pathogens are expressed in plants, and their utility as immunogens/therapeutic proteins is investigated. However, their widespread application is impeded by low levels of expression, variability, and insufficient immune responses after oral administration together with insufficient information on the possible deleterious effects due to their use in the long run. Recent studies have answered some of these problems, and more research is in progress to bring these transgenic plant products into clinics. On the other hand, there are several reports on their efficacy both in experimental animal models and in human beings. Here in this chapter, we have discussed about the recent progress with respect to the expression and use of plant-derived vaccine antigens. Although the recent work in this direction provides enough information, yet there is lot of scope for improvisation.

13.1 Introduction

In the present-day world, existing and continuously emerging diseases together with the problems associated with the available vaccines, like safety and poor efficacy, are challenging the researchers to develop more efficient, highly economical, and

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easily transportable vaccines that can be administered, preferably, by simple feeding without the requirement of any trained personnel. "Prevention is better than cure" is the strongly held notion among the health departments across the world, and this seems possible only by vaccinating the population under threat, although vaccination is not the universal solution. With almost 200-year-long research in vaccinology, we could eradicate smallpox; the polio is almost consigned to history and much more are in the pipeline. Practically, an ideal vaccine should be able to protect against multiple infections and should be economical, resistant to heat, safe, and easy to produce. Till date, no such vaccine has been made, although the research is progressing step by step toward this goal and the results are encouraging. However, most of the currently available vaccines are associated with several problems including safety due to vaccine-associated side effects in human subjects (Eldred et al. 2006; Plesner 2003; Segal et al. 2006).

Since the molecular biology of the union between *Agrobacterium tumefaciens* and plants is unraveled, the plant biotechnology has grabbed a lot of attention from both plant and animal biologists (Drummond et al. 1977). On one hand, plant biologists are busy developing high-yielding and disease-resistant transgenic varieties by introducing the desired traits into agronomically important plants (Sakamoto and Matsuoka 2004). On the other hand, animal biologists are exploiting this system for the production of safe and highly economical proteins of therapeutic use, including vaccine antigens (Fischer et al. 1999; Goldstein and Thomas 2004; Ruf et al. 2001; Tripurani et al. 2003). Transgenic plant technology has already proved its importance as a tool for the production of therapeutic proteins of human and animal use (Langridge 2000). However, the efforts to bring this technology close to clinics are hindered by some of the practical problems and the foreseen threat, based on scientific assumptions and public fear, due to these genetically modified (GM) plants. Here, in this chapter, we have discussed the practical problems associated with this technology and the recent developments made either to overcome these problems or in finding alternative approaches, with a special emphasis on plant-based vaccines.

13.2 Basic Concept of Edible Vaccines

The concept of edible vaccines was materialized when tobacco was used to express hepatitis B surface antigen to produce an immunologically product (Mason et al. 1992). This showed scientists new avenues to use the technology for the production and application of edible vaccines and antibodies for immunotherapy especially in developing countries like India, Africa, etc. The desired gene of interest is introduced into the selected plants to manufacture proteins for oral administration in the humans or animal body. These are generally subunit preparations where they are engineered to contain antigens but bear no genes that would enable whole pathogens to form. It assures safety against establishing infection, especially in immunocompromised patients.

Plants serve as efficient factories with a low amount of cost inputs. Their production is highly efficient and cheaper in terms of cost for field grown plants are lower as

compared to in vitro (Xu et al. 2011). Plant-derived vaccines are heat stable and eliminate the need of cold chain storage saving the money spent by pharmaceutical companies to preserve vaccines (Nochi et al. 2007). The plants can be adapted to the ecological condition to avoid long-distance transportation, storage and can be easily scaled up (Lal et al. 2007). In comparison to conventional subunit vaccines, edible vaccines are more compliant, especially in children. Oral administration eliminates the requirement of syringes and needles, a trained medical personnel, and large capital intensive pharmaceutical manufacturing facilities (Webster et al. 2002b; Lal et al. 2007). Transgenic plants have low contamination risks as compared to injected vaccines.

An edible vaccine with multicomponent capability is achievable due to the development of hybrid plants through transgenic technology. These second-generation edible vaccines can be used against multiple diseases as they allow several antigens to prime M cell (microfold cell) simultaneously (Lal et al. 2007). Thus these edible vaccines could be more effective than injected vaccines (Ramessar et al. 2008a, b; Naqvi et al. 2011).

13.3 Plants as Expression Systems for the Recombinant Proteins

The edible vaccines are plant-produced recombinant protein vaccines which induce protective immunity against the specific animal pathogens. These plants are capable of producing specific antigen(s) through genetic engineering and deliver the antigen when plant part/whole is consumed orally (Lossl and Waheed 2011). The attention on edible vaccines was increased rapidly after successful development of genetic transformation of tobacco in the year 1983, when plants were almost immediately prospected for production of various recombinant proteins (Golovkin 2011) (Fig. 13.1). As compared to the bacterial expression systems, plants offer many advantages for their use as expression systems such as the presence of eukaryotic posttranslational modification machinery, the safety of use of plant-derived products in humans or animals, and being free from the contamination of preparations from bacteria (Yusibov and Mammedov 2010). Glycosylation is an important post-translational modification, and mammalian glycoproteins have been shown to get glycosylated while getting expressed in transgenic plants. Sometimes the glycosylation of these biopharmaceuticals, however, is not performed properly in plant systems due to the structural dissimilarity between the two systems, namely, plant complex N-linked glycans which contain β1,2-xylose and α1,3-fucose residues which are absent in human glycans. To overcome this problem, humanization of N-linked glycosylation and N-linked glycans of biopharmaceuticals has been done in the recombinant proteins expressed in plants (Saint-Jore-Dupas et al. 2007; Frey et al. 2009; Vezina et al. 2009; Matsuo and Matsumura 2011). The transgenic approach of recombinant protein production in plants has been employed in both whole growing plants and plant cell cultures via conventional fermentation (Boehm 2007; Floss et al. 2007).

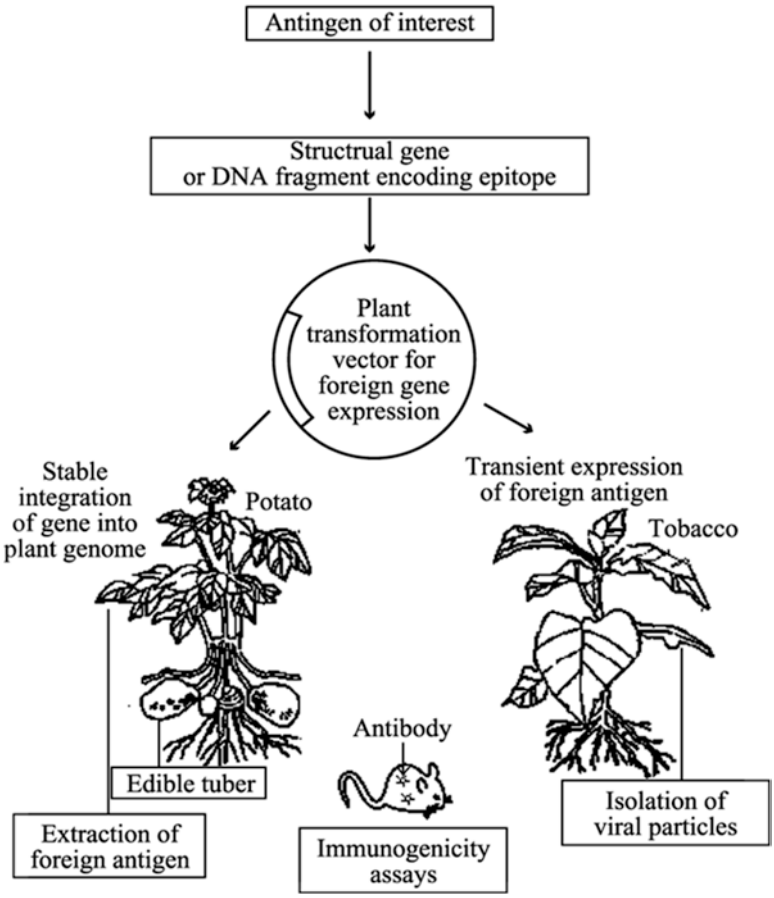

Fig. 13.1 Process of producing transgenic plant vaccines (Taken from Han et al. 2006; revised from Mason and Arntzen 1995)

13.3.1 Transformation Methodology Used for Edible Vaccines

For many years, *Agrobacterium*-mediated transformation of plants was restricted to those susceptible to natural infection, particularly to the dicotyledonous plants. Enormous amount of research in this field of biotechnology over the last two decades resulted in the development of protocols for the transformation of previously non-susceptible agronomically important plant species including monocots (Cornejo et al. 1993; Gelvin 2003b; Jayashree et al. 2003; Anuradha et al. 2006; Sharma et al. 2006; Venkatachalam et al. 2006). *A. rhizogenes*, a species of *Agrobacterium* generally used in the transformation of root cultures, can also be used (Christey and Braun 2005). *Agrobacterium*-mediated transformation results in integration of the transgenes into plant cell genome, and this method has been

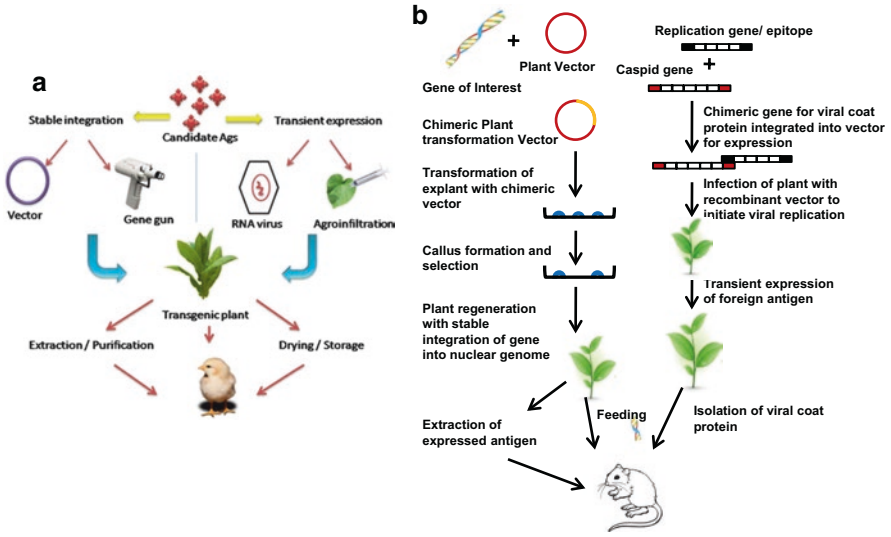

Fig. 13.2 (**a**) and (**b**) Strategies for the production of candidate vaccine antigens in plant tissues

widely used due to its merits: simple to perform, having higher transformation efficiency due to less stringent selection procedures, providing a suitable environment for complex posttranslational modifications, subcellular targeting, etc. This procedure, however, is associated with low levels of transgene expression and some environmental hazards. Thus, the expressed protein in transgenic plants might not always serve the purpose, particularly when these plants are developed for oral vaccination where a large amount of recombinant protein is required.

The other possibility is to transfer the gene of interest into chloroplast by particle bombardment using gene gun. The chloroplast transformation vectors carrying the gene of interest are coated onto micro-projectile particles and accelerated into plant tissues (Daniell et al. 2005; Ruf et al. 2001) (Fig. 13.2). The advantages of this system include high-level expression of transgenes and integration at defined positions and rule out the possibility of transgene silencing. Most of the mammalian proteins, however, undergo complex posttranslational modifications. These modifications determine their activity and overall intracellular stability. Chloroplast system, being prokaryotic in nature, doesn't have the machinery to perform these complex modifications. This makes it less potential than the nuclear transformation, particularly when posttranslational modifications are indispensable. Also, due to the stringent selection procedures involved in this method, the transformation efficiency is comparatively very less. Many groups are working to overcome these problems to make this system more suitable for molecular farming.

The plant expression is, however, not a straightforward practice, and each gene has to be tested case by case before planning for large-scale production. In such cases, methods described above are not the most preferred, as they require too much

time to establish the suitable conditions. Recently, many transient expression models have been proposed for analyzing the recombinant plant expression constructs (Baur et al. 2005; Lorkovic et al. 2004; Kumar et al. 2005; Marillonnet et al. 2005; Huang et al. 2006; Varsani et al. 2006). A study using "launch vector" system showed that plant-derived antigens of influenza virus are highly immunogenic and protected ferrets against the viral challenge (Mett et al. 2008). The launch vector has the advantageous features of standard agrobacterial binary plasmids as well as plant viral vectors, to achieve high target antigen expression in plants. This new approach has been employed for the production of a wide variety of target antigens from several pathogens including *Bacillus anthracis*, *Yersinia pestis*, *Influenza virus*, *Plasmodium falciparum*, *Trypanosoma brucei*, and measles virus in *N. benthamiana* plants (Musiychuk et al. 2007). Domain 4 of PA from *B. anthracis* was successfully produced as a fusion protein with a thermostable carrier protein derived from *Clostridium thermocellum* (Musiychuk et al. 2007). Although there are many methods for simple analysis of recombinant protein expression, only some of them are suitable for studies where decent amount of protein is required for functional assays, like *Agrobacterium* infiltration (Huang et al. 2006), recombinant plant viral expression (Varsani et al. 2006), and suspension cell culture-based (Kumar et al. 2005) expression systems. In particular, when a vaccine antigen is to be tested, these methods are highly useful, and within few weeks enough protein can be produced for immunogenicity experiments. Once tested at a smaller scale, and if considered suitable for oral vaccination, either of the above standard methods can be opted for large-scale production. Thus, the transgenic plants are then screened to pick the suitable ones for scale-up. Considering the advantages mentioned earlier for chloroplast system, if glycosylation is dispensable, transplastomic plants can be developed for scale-up procedures. These plants express comparatively higher amounts of recombinant proteins per unit area, a factor that is more desirable in a plant-derived vaccine (Lelivelt et al. 2005; Zhou et al. 2006).

13.3.2 Genetic Aspects

When a plant is infected with *Agrobacterium*, the vir gene products of the bacterium are involved in the processing and further transport of the T-DNA region, carrying the gene of interest and the selection marker across the plant cell wall, and guide the T-DNA into the plant cell nucleus, where the T-DNA is randomly integrated into the plant chromosomal DNA (Gelvin 2003a). The transgene is, then, expressed in the plant cells and is processed in a similar fashion as it is processed in its native cell environment. However, care needs to be taken while designing the expression construct to avoid any unwanted effects on the transgene expression. Some of the critical issues relating to this aspect are discussed in the following sections.

13.3.2.1 Integration of Vector-Derived Sequences

Nuclear transformation of plants is known to be associated with transgene silencing due to many factors including the integration of superfluous vector DNA-derived sequences (Kononov et al. 1997). All of the plasmids used in plant transformation technology contain bacterial sequences, and these are known to induce strong transgene silencing, a plant defense mechanism developed against invading microbes. For this reason, after *Agrobacterium* transformation, resultant plants are extensively screened for those that do not carry the vector backbone sequences. This is cumbersome and often requires screening as many as 100 transgenics to obtain the desired transgenic plant. *Barnase* gene product is toxic to plants, and, hence, inclusion of this gene just outside the T-DNA borders serves as an easy screening strategy at the transformation stage itself to avoid the incorporation of superfluous DNA sequences into plant genome (Hanson et al. 1999). This strategy can also rule out possible unintended leakage of harmful vector-derived antibiotic resistance markers into the environment.

13.3.2.2 DNA Methylation

Methylated DNA, in combination with a repressor complex, causes change in the chromatin structure, and these chromatin structures have been shown to be transcriptionally inert (Mishiba et al. 2005). The repressor complex includes histone deacetylases (HDACS) that remove acetyl groups from the amino-terminals of the lysine residues of the core histone proteins. Genes that are associated with the deacetylated histones are not accessible to the transcription machinery. Thus, methylation of chromatin changes the acetylation status of the surrounding histones and, in association with the chromatin remodeling proteins, influences the active state of the chromatin, making it transcriptionally inert. Particularly in plants, gene silencing mediated by DNA methylation seems to be global. This mechanism has been implicated as a plant defense mechanism against the invading microbes and transposable elements. There are many occasions where methylation of promoter DNA has caused silencing of the (trans)gene expression (Fagard and Vaucheret 2000). DNA methylation can induce both TGS and PTGS machinery of plants. DNA methylation of promoter region causes activation of TGS machinery, whereas methylation of coding region causes induction of PTGS machinery. Transgene-specific RNA-mediated DNA methylation followed by gene silencing has been reported to occur in transgenic plants (Zilberman et al. 2004). Thus, established silencing can induce shutdown of even endogenous gene expression, a process known as homology-dependent gene silencing or co-suppression (Rocha et al. 2005). There are many examples of co-suppression in plants, and this effect spreads systemically in the plant. T-DNA insertion at more than one position in the plant genome is one of the major factors that contributes for DNA methylation in transgenic plants (Muskens et al. 2000). Recently silencing of transgenes in all the transgenics due to methylation of 35S Cauliflower mosaic virus promoter in gentian plants and the use

of different promoters resulted in the expression of these transgenes which has been reported (Mishiba et al. 2005). Thus, DNA methylation plays a major role on the overall expression levels of gene products.

13.3.2.3 Promoter Elements

Promoter elements, present just upstream of all open reading frames (*orf*s), are highly indispensable for the expression of any gene and are the main driving force that determine the overall levels of gene products in the cell. An active promoter transcribes the genes into mRNA transcripts, which will be translated into proteins by ribosomes. The presence of an efficient promoter results in the accumulation of large amounts of mRNA transcripts and their gene products in the cell. Most commonly, the gene of interest is cloned under the CaMV 35S promoter for nuclear transformation, which results in constitutive expression of the desired gene product. However, CaMV 35S promoters or any dicot-specific promoters are found to be less efficient in monocots, and, thus, a monocot-specific promoter, like maize ubiquitin-1, is suggested for expression in monocots (Cornejo et al. 1993). Although the use of constitutive promoters provides the researcher with the advantage of extracting the protein from the desired plant tissue for analysis, it is realized that constitutive expression of recombinant proteins affects the plant growth and the protein stability. Thus, targeting the expression of recombinant protein to a specific site, depending on the expression host and purpose of the transgene product, by using the tissue-specific promoters is the most preferred practice (Al Babili et al. 2006). This strategy minimizes the recombinant protein toxicity on the whole plant, avoids the risk of possible negative effects on nontarget species, and also increases the stability of expressed protein. For example, when oral vaccination is the ultimate purpose, the use of tuber-specific promoter for the expression of vaccine antigen in a host-like potato should be preferred (Lauterslager et al. 2001)**.** Using this strategy, expression strategies can be designed depending on the purpose and also transgene-associated risks on the nature. Like if the transgene containment is highly essential, the transgene can be driven under a promoter specific for any vegetative part, and these parts can be harvested just before the beginning of reproductive phase, particularly in biannual plants, to avoid pollen-mediated gene flow to unintended plants. In case purity of the therapeutic protein is crucial, seed-specific promoters can be used to drive the transgene tagged to purification-assisted genes for which easy and efficient purification protocols are established, as is the case for oleosin-based fusion protein purification strategy (Parmenter et al. 1995).

On the other hand, if the stability of the expressed protein in the host cell environment is the main concern, this problem can be overcome by using an inducible promoter, for example, tomato hydroxy-3-methylglutaryl CoA reductase 2 (HMGR2) promoter, a mechanical gene activation system (MeGA) in that the gene

under the influence of this promoter is expressed during the tissue harvesting (Ma et al. 2003). This approach also reduces the problem of recombinant protein toxicity on the plant and helps in normal plant growth. There are many such inducible promoters that can be used in transgenic plant research (Lee and Yang 2006; Padidam 2003).

13.3.2.4 Codon Optimization

Although all the proteins are made up of same set of amino acids, different cell systems show varying affinities for triplet codon coding (Al Babili et al. 2006; Karasev et al. 2005; Muller 2005). These differences in the affinities for different codons, coding for a particular amino acid, result in either a pool of truncated proteins or production of completely different proteins due to frameshifting, when a heterologous system is considered for expression. This has direct relation to the efficiency of translation and, thus, has a direct effect on the overall recombinant protein levels in the cell, for example, both *CAU* and *CAC* codes for histidine. But plant cells may show stronger affinity for *CAC*, and the same codon may be less preferred in mammalian cells. Thus, when heterologous gene is intended for expression in a plant system, in general, lower expression levels are observed. However, it is observed that when the gene is optimized according to the specific plant codon usage, at least tenfold increase in the expressed protein levels is observed (Horvath et al. 2000; Karasev et al. 2005). For this, either the whole gene can be optimized according to the codon biasing of the host cell system or only those codons can be replaced, which are rarely used in the intended expression host. This can be achieved either by PCR amplification of multiple synthetic oligos coding for the gene or by simple chemical synthesis of the entire gene. If only few bases need to be changed, this can be achieved by site-directed mutagenesis of the specific gene. In plants, most of the endogenous genes are rich in G+C; hence, genes with high A+T bases are poorly expressed. However, codon optimization seems to improve the expression levels under certain conditions only. It has been reported in a comparative study that codon optimization would increase expression levels and the levels are higher when the optimized gene is driven under the influence of an optimal promoter. Similarly, when a less modified gene is driven under the influence of a stronger promoter and a stronger enhancer, like enhanced 35S CaMV promoter and AMV enhancer, respectively, there would have more positive effect on expression compared to the expression levels obtained with a heavily optimized gene driven under the influence of relatively stronger promoter and enhancer elements (Suo et al. 2006). These results suggest that the effect of codon optimization on expression of recombinant proteins in plants depends on the promoter and enhancer elements used to drive their expression.

13.3.3 Transcriptional Aspects

Levels of transcript in the cell depend mainly on the promoter under which expression of the gene is driven. In case of transgenic plants, generally CaMV 35S promoter or its enhanced versions are used as constitutive promoters, which results in the expression of transgenes in all parts of the plant. Theoretically, under suitable expression conditions, any transformed plant would produce sufficient amounts of recombinant protein. However, practically this doesn't seem to be the case, and, in some instances, expressing heterologous genes in plants has often been associated with gene silencing due to several factors. Recently, several studies have indicated that these problems can be overcome by taking precautions while designing the expression construct, some of which have been discussed in the previous sections. Here, we discuss about those factors that need attention at the transcription level.

Plants are known to possess different transgene silencing mechanisms as part of their defense against the invading microbes and foreign genes. Thus, further investigations into transgene silencing would provide information on different mechanisms controlling plant genome structure and gene expression. Transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) are the frequently identified mechanisms involved in transgene silencing. The above mechanisms reflect alternative responses to two important stress factors that the plant's genome undergoes: integration of additional DNA into its chromosome and extrachromosomal replication of viral genome using the cellular machinery. There are several reports available in the literature illustrating either of these mechanisms in transgene or endogenous gene silencing.

13.3.3.1 Intron-Mediated Enhancement of Transcription

Strong promoters, like 35S promoter of CaMV in plants and *Cytomegalovirus* (CMV) in animal cells, are very efficient in increasing the transcript levels of genes driven under these promoters. However, it is learned that inclusion of an intron together with these strong promoters significantly enhances the transcription rate and thus influences the total recombinant protein yields (Mascarenhas et al. 1990; Rethmeier et al. 1997; Rose 2004). These intron regions carry sequences that are similar to those found in the untranslated regions (UTRs) of the genes and are believed that they are crucial for the observed activity. Apart from enhancing the transcript levels, they have also been found to increase the translation levels of the genes (Clancy and Hannah 2002; Jeong et al. 2007). Moreover, it is found that placing introns close to promoter regions is crucial in their activity. Thus, introducing introns into the construct, particularly toward the promoter region, would help in increasing the functional mRNA transcripts as well as the gene products in the transformed cell.

13.3.3.2 Transcriptional Gene Silencing (TGS)

TGS results in impairment of transcription and can be triggered either in *cis* or in *trans.* This state of transcriptional silencing results basically through promoter DNA methylation and/or chromatin condensation. Endogenous heterochromatin around the transgene locus, endogenous methylated repeated sequences located in the near vicinity of transgene locus, transgene-genomic junctions that alter chromatin organization, and specific spatial arrangement of transgene repeats that create heterochromatin locally are the *cis*-acting elements that influence the active state of the chromatin around the transgene locus, thereby leading to the establishment of TGS (Iglesias et al. 1997; Prols and Meyer 1992; ten Lohuis et al. 1995; Ye and Signer 1996)**.** *Trans*-acting elements could be allelic or ectopic homologous (trans)gene loci that transfer their epigenetic state through direct DNA-DNA pairing or ectopic transgenes or RNA intermediates of DNA viruses (Al Kaff et al. 1998; Luff et al. 1999; Mathieu and Bender 2004; Mette et al. 1999; Thierry and Vaucheret 1996). In some, but not all, the state of transcriptional silencing is released when these loci are expressed either in the methylation-deficient *ddm2* mutants or in plants expressing antisense RNA to *DDM2* transcript that codes for DNA methyltransferase (Mittelsten et al. 1998), whereas all the silenced genes were reactivated in *ddm1* mutants that are deficient for a protein that influences transcriptional activity and the chromatin state. In a study conducted in the genetic model *Arabidopsis* to look for the endogenous targets of TGS, it was found that a class of repetitive elements localized in the pericentromic regions, known as transcriptionally silent information (TSI), were responsible, as the transgenes are readily expressed in *tsi* mutants, but not in PTGS-negative mutants (Steimer et al. 2000). A further insight into this process revealed that methylation of promoter DNA was only responsible for the observed transcriptional silencing. Furthermore, there are instances where transgene silencing is observed even among transgenics containing a single copy of the transgene (Iglesias et al. 1997; Prols and Meyer 1992). Finally, TGS is both mitotically and meiotically heritable in plants and affects only sequences integrated into the genome, but doesn't influence extrachromosomal nucleic acids, like those of DNA viruses.

13.3.3.3 Posttranscriptional Gene Silencing (PTGS)

RNA interference, a dsRNA-mediated gene regulation mechanism, is known to exist in all living organisms, and the participation of a dsRNA-cleaving dicer/dicer-like enzyme has been implicated in this process. Dicer/dicer-like enzyme cleaves the dsRNA into small 21–25 nt fragments, known as small interfering RNA (siRNA) molecules. These siRNAs are implicated to be involved in the silencing of gene expression by cleaving the target mRNA in association with the RNA-induced silencing complex (RISC). A similar mechanism has been reported to be involved in transgene silencing in plants at the posttranscriptional level and is known as

posttranscriptional gene silencing (PTGS). In plants, PTGS was first demonstrated in *Petunia*, in which introduction of transcribed sense transgenes into *Petunia* resulted in the silencing of homologous endogenous gene expression (Napoli et al. 1990) and the silencing has been shown to occur at the posttranscriptional level through degradation of both endogenous and transgene RNA. In PTGS, as in TGS, several silencing sources cause the activation of RNA degradation machinery. They can be the transcripts from a sense transgene, an antisense transgene, either sense and antisense transcripts or viruses. In some cases, PTGS has been linked to methylation of coding sequence, although methylation status alone doesn't influence the activation of RNA degradation machinery (Jones et al. 1999; Nolan et al. 2005). These results also emphasize that methylation of coding sequence in some of the PTG-silenced plants could be an indirect consequence. In PTG-silenced plants, PTGS can occur in *cis*, in *trans*, or in both *cis* and *trans*. Unlike in TGS, in PTGS, the silencing effect can be imposed upon extrachromosomal sequences like viral genes (Dougherty et al. 1994; English et al. 1995). It is found that transcription of transgenes precedes their methylated state, which means that an RNA molecule is involved in imposing methylated state on the transgene. Methylated transgenes are known to produce aberrant or truncated RNA, which can act as sources of PTGS (Jones et al. 1998; Wassenegger and Pelissier 1998).

On the other hand, integration of vector backbone sequences into plant cell genome during transformation results in a state of transgene silencing. On detailed investigations into this aspect, it was found that the presence of inverted repeats in the transferred sequence was responsible for the observed silencing effect. PTGS has been shown to occur in transgenic plants carrying transgene inverted repeats on several instances (Hamilton et al. 1998; Ma and Mitra 2002; Stam et al. 2000). Silencing is also induced when there are multiple integrations of the transgene in the genome, in which case levels of transgene mRNAs above the threshold cause the induction of silencing (Lechtenberg et al. 2003). In all these silenced plants, small double-stranded RNAs of approx. 21–25 nt that are characteristic of dsRNA-mediated RNA interference can be isolated. The occurrence of PTGS in transgenic plants can be identified by performing a simple northern hybridization for smaller RNAs. Insights into this mechanism in combination with studies on some of the plant viruses' biology and their survival in the plant cells resulted in the identification of viral gene products that suppress the activation of PTGS against the transgene (Bisaro 2006; Silhavy et al. 2002; Zhao et al. 2005). Characterization of these PTGS suppressors indicated that these are primarily the pathogenicity determinants encoded by the viral genomes during the infection process (Brigneti et al. 1998). Thus, co-expression of these suppressor genes along with the transgene under study or simple crossing of silenced transgenic plants with those expressing the viral suppressor gene products can rule out PTGS-mediated transgene silencing in transgenic plants. However, recently there are reports illustrating the persistence of transgene silencing in the plants even in the presence of viral suppressors, which may not be universal, suggesting the existence of strong antiviral defenses in plants (Li et al. 1999).

13.3.3.4 mRNA Stability

Apart from PTGS-directed transgene mRNA degradation, it is also found that mRNA degradation occurs due to RNA instability sequences, which too can greatly influence the accumulation of recombinant protein in the cell. In general, the stability of the transcript depends on their 3′UTRs (Akashi et al. 1991), 5′cap, the length of 3′ poly(A) tails (Gallie 1991), base composition of the transcript, and, sometimes, even on the host used for expression. Specific 3′UTR instability motifs (AUAGAU and GAU motifs) have been identified in plants and are found to be responsible for the observed instability (Newman et al. 1993) and are common to all those unstable mRNAs. In yeast, it is found that the presence of shorter 3′ tails is associated with removal of 5′ cap, and de-capped RNAs are susceptible to 5′3′ exonuclease degradation (Decker and Parker 1993; Mandart et al. 1994). Bt-toxin transcripts are rich in A/U sequences and are usually stable in the bacterial cell. It is found that these transcripts are highly unstable when expressed in *Arabidopsis*, whereas conversion of A/U to G/C sequences synthetically enhanced stability in the same plant, indicating that A/U-rich sequences are highly prone to degradation in plants. However, in a comparative study between *Arabidopsis* and tobacco, it is observed that some of the sequences that limit the mRNA accumulation in *Arabidopsis* did not show any effect when expressed in tobacco system. This suggests that the stability of mRNA also depends on the host cell, in which they are expressed (De Rocher et al. 1998). Conversely, another report acknowledges the importance of AUUUA motif in the RNA and also concludes that high A/U content is not purely responsible for the mRNA decay (Ohme-Takagi et al. 1993). Usually, these instability sequences can be removed either by mutagenesis or by in vitro synthesis of the coding region. Despite abovementioned developments, more work needs to be done to better understand problems associated with transcriptional gene silencing and mRNA degradation and also to look for logical answers to overcome these problems.

13.3.4 Translational Factors

If the transgene processing is not affected until the synthesis of a stable mRNA transcript, then the transcripts are translated into polypeptides. These newly synthesized polypeptides undergo extensive processing before becoming mature and functional proteins. In eukaryotic cells, usually the 5′ end of the mRNA is capped, and the 3′ end possesses a poly(A) tail, both of them being highly indispensable for efficient translation. The efficiency of translation also depends on factors like the presence of leader sequence ahead of first ATG (the start codon), availability of Kozak sequence (GCCA/GCCATGG), the distance between the first codon and the cap, etc. (Kozak 1991). Thus, these general rules must be considered while designing the expression constructs. Considering the requirement for large amounts of protein for oral vaccination, edible vaccine production in plants requires

accumulation of maximum protein per unit area. However, this seems difficult due to several reasons that include the following factors, particularly when expressing in a heterologous system.

13.3.5 Translational Enhancers

The coding region of all the genes is usually flanked by noncoding regions (NCRs) that play an important role in determining the levels of expression of the respective protein. These NCRs provide optimal conditions necessary for ribosomal binding, while the ribosomes screen for the start codon and also play a major role in mRNA turnover. Their efficiency lies in their length, number, and strength of secondary structure upstream and downstream to first start codon, the context around the start codon, and so on. In a 5′NCR, Kozak (GCC(A/G)CCAUGG) sequence provides the optimal context for efficient translational initiation. Mutation studies around the first start codon revealed that adenosine at -3 position and a guanosine at the +4 position from A (+1 position) of the start codon have a strong positive effect on the translation. Apart from this, there are many other factors (Kozak 1991) that make the NCR a good enhancer of translation. There are some well-characterized 5′NCRs that act as efficient translational enhancers, and inclusion of these NCRs in transgene expression constructs has been shown to increase transgene expression dramatically. For example, *Tobacco etch virus* 5′ leader has been used extensively as an efficient translational leader for plant-based expression of many bacterial and viral genes of clinical significance. This leader is found to be active even in mammalian cell systems and promotes cap-independent translation of associated genes in the presence of several translation initiation factors. The efficiency of these NCRs, weak or strong, is determined by their activity in the presence of minimum levels of translational factors. Similarly, 5′ NCRs of *Alfalfa mosaic virus* and *Tobacco mosaic virus* have been used extensively as translational enhancers (Browning et al. 1998). Recently it has been found that sequences in the noncoding regions flanking the coding regions that are similar to a part of 18S rRNA sequences influence the translational rate. This effect could be seen even when such sequences are introduced into the mRNAs at the inter-cistronic regions and the efficiency increases with the increase in the number of such interrupted sequences, but to some extent (Akbergenov et al. 2004). This is a growing field, and several such studies are in the pipeline to understand the mechanism of their action. However, this translational enhancing effect may not be universal, and the impact of these sequences on the translation of genes has to be studied case by case. On another hand, 3′ NCRs also play a crucial role in translation, and poly(A) tails play a crucial role in stabilizing the ribosomal binding to the 5′ end of the transcript.

13.3.5.1 Signal Sequences

Generally, endogenous gene products of plant cells are either directed to the secretory pathway or retained in the cytoplasm, if the protein is destined for cytosol. Those directed to the secretory pathway are usually tagged with an N-terminal signal peptide that helps in guiding the premature form (preprotein) of the expressed protein to the secretory pathway. Most likely, all the mammalian genes coding for secretory proteins carry their own targeting signals. However, plant-derived signal sequences can also be fused to the transgenes for efficient targeting (Richter et al. 2000; Schaaf et al. 2005). Once the expressed preprotein enters the secretory pathway, it undergoes several modifications before it is either secreted into the apoplast/plasma membrane, or translocated to the specific compartment as guided by its targeting signal (Nassoury and Morse 2005). Thus, based on the purpose, the transgene products can even be targeted to desired subcellular compartments using the specific plant signals. It is believed that endoplasmic reticulum (ER) targeting of transgene product, like vaccine antigen, is safer due to lower levels of proteases and the retained protein is properly folded in the presence of heavily loaded chaperones in the ER (Haq et al. 1995; Huang et al. 2001). In most cases, transgenes have been tagged to ER retention signal peptides (KDEL/HDEL) that results in higher accumulation of the recombinant protein in a more stable and concentrated form in the ER (Huang et al. 2001; Wandelt et al. 1992). This approach is highly significant in the context of edible vaccine production, both in terms of increasing the concentration of the expressed protein and in protecting it from the cellular proteases. It has been reported that some amount of retained proteins is secreted through the roots (rhizosecretion) into the outer environment and recently many recombinant proteins have been recovered from plants grown in hydroponic medium (Borisjuk et al. 2009). When edible vaccine production is considered, ER retention seems to be the most suitable approach to increase the amounts of recombinant vaccine antigens per unit biomass. ER retention strategy is also applicable where suspension cell cultures are used for transgene expression, in which case the recombinant proteins can be recovered from pooled cells by gentle enzymatic lysis followed by chromatographic purification. However, no purification is necessary for the plant-expressed vaccine antigens. Cells expressing the recombinant vaccine antigens can be directly considered for oral vaccination, where these cells play dual roles of protecting the antigens from the acid environment of the stomach and also acting as adjuvants. If extraction and purification of the recombinant protein are the aim, as is the case for therapeutic proteins, transgenes can be fused to purification-assisted protein tags (Parmenter et al. 1995; Valdez-Ortiz et al. 2005) and then purified from the respective compartment using the simple standard protocols. However, some of these approaches are applicable either for small-scale production of recombinant proteins or for the production of therapeutic proteins and do not serve any purpose in the large-scale production of edible vaccines.

13.3.5.2 Posttranslational Modifications

Posttranslational modifications include glycosylation, intracellular translocation of the protein, protein folding, and so on, and these modifications strongly influence the physicochemical and biological properties of proteins. All those proteins that are targeted to secretory pathway undergo several modification events before being translocated to their destinations. Not many differences are found between plants and mammals with respect to posttranslational machinery. Both the systems follow almost the same set of rules while processing the expressed protein. However, it is important, particularly in the context of edible vaccine technology, to discuss about the important differences that exist between these two systems, at least to evaluate the advantages and disadvantages of plant-derived products in animal and human therapy.

13.3.5.2.1 Glycosylation

Glycosylation, the addition of carbohydrate side chains onto a nascent polypeptide, strongly influences the intracellular distribution and overall stability of the expressed protein in a cell. Most of the therapeutic proteins are functionally active and are highly stable in their glycosylated forms, and, thus, it is necessary to understand the glycosylation pathways in plants involved in the processing of heterologous proteins used in human therapy. As mentioned earlier, a protein destined to secretory pathway carries an N-terminal signal that directs the newly synthesized protein to the ER. Further maturation, to a functional protein, takes place, while the proteins pass through the secretory pathway, starting from ER all the way to Golgi complex, where different glycans are either added to or removed from the polypeptide backbone. Extensive research in this field of plant biotechnology revealed that plants and mammals do not differ much with respect to their early posttranslational modification events. However, it is found that complex N-glycan maturation events in the late Golgi apparatus result in the addition of core $\propto$(1,6)-fucose and β(1,4) N-acetylglucosamine in case of mammals, whereas in plants core $\propto$(1,3)-fucose and bisecting β(1,2) xylose are added, respectively (Faye et al. 2005). It is also found that, in plants, β(1,3)-galactose and $\propto$(1,4)-fucose linked to the terminal N-acetylglucosamine form *Lewis-a*-type oligosaccharide structures instead of β(1,4)-galactose in combination with sialic acids, as seen in mammals (Gomord et al. 2005). Interestingly, plant-specific N-glycans are found only on the proteins extracted from the older leaves, whereas high-mannose-type glycans that are typical for mammalian proteins are seen on the proteins extracted from young leaves (Elbers et al. 2001). On the other hand, incorporation of these plant-specific immunoreactive glycans can be completely avoided either by directing the recombinant proteins for ER retention using HDEL/KDEL sequences at their C-terminal end or by modifying the purified proteins, the latter being practically not relevant in the context of edible vaccine production.

Attempts have been made to express and target the animal glycosyltransferases in plants to produce mammalian-like proteins. Thus, transgenic plants are made to successfully express the heterologous glycosyltransferases and converting the plant-made mammalian proteins into mammalian-like proteins (Bakker et al. 2001, 2006). However, sialylation of recombinant proteins was still unachievable due to the fact that plants do not possess the basic biochemical machinery for sialylation. Contrary to this, some workers claimed for the occurrence of sialylated endogenous proteins in plant cells and argued that in vivo sialylation is very much possible in plant cells (Shah et al. 2003). Altogether, this still remains a matter of long debate and is, thus, open for further investigation. Considering the currently available information, if sialylation of the recombinant protein is indispensable for its activity, then either all the enzymes involved in sialylation need to be co-expressed with the desired transgene or it can be performed for purified proteins as mentioned above. However, if sialylation of vaccine antigen is crucial for its activity, then production of sialylated vaccine antigen in plants for oral vaccination poses a huge challenge.

On the other hand, very little is known about the O-glycosylation events in plants, and this area needs more focus to understand the possible effects of this event on the activity and stability of recombinant proteins expressed in plants (Faye et al. 2005). This will also enable us to study the possible deleterious effects of any such plant-specific O-glycans on human and animal health, although no such events are expected as all mammals consume plants/plant products in their daily diet.

13.3.5.2.2 Protein Stability

Proteins, while they pass through the secretory pathway, also undergo proteolytic processing before being translocated to their final destination in a more stable and active form. Proteolytic processing of the immature protein is essential for it to become a mature, stable, and functionally active protein. This process involves the following events: (i) trimming off their signal peptides by signal peptidases at the ER, (ii) several modifications in the presence of a pool of proteolytic enzymes along the secretory pathway, (iii) stabilization and folding into a highly structured protein in the presence of chaperones and their targeting peptides, and (iv) translocation and finally removal of the targeting peptides by a family of proteinases, known as pro-protein convertases, in their respective cellular compartments. Proteins without subcellular targeting signals are finally secreted into apoplast/plasma membrane from where they are secreted into extracellular space. Overall amounts of the recombinant proteins depend not only on their expression levels but also on the stability of the protein during the process of transport across the secretory pathway. Several laboratories have reported extensive proteolytic degradation of recombinant proteins along the secretory pathway, particularly in the leaf tissues of vegetative plants, where the expressed proteins are present in a highly aqueous environment (Outchkourov et al. 2003; Sharp and Doran 2001; Stevens et al. 2000). Glycosylation status of these proteins greatly influences their intracellular stability and susceptibility to the proteolytic enzymes. Although proper glycosylation results in more

stability, directing the recombinant proteins to the ER has been proposed to be the safest option to avoid undesired degradation. The proteins requiring extensive post-translational processing are fused with the factors that improve their intracellular stability without interfering with their basic conformation (Dolja et al. 1998; Graslund et al. 2002; Nilsson et al. 1997). Some of these fusion partners also assist in downstream purification processing of fused proteins, if required (Graslund et al. 2002). Another option is to specifically express the recombinant proteins in the seeds, where the proteins are highly stable for longer periods of time. However, when edible vaccine production is considered, application of this approach needs to be given a thought, as most of the seeds are palatable only after cooking. On the other hand, production of transgenic hosts expressing desired protease inhibitors or those with reduced levels of proteases will be very handy in transgenic technology for the production of safe and highly stable vaccine antigens in transgenic plants.

13.3.6 Production Hosts

Charles J. Arntzen, in the early 1990s, studied the possibility of expressing vaccine antigens in plants for developing edible vaccines using plant biotechnology, with a vision in mind that 1 day millions of children will get immunized to those dreadful diseases through their daily diet, instead of taking painful needle-based vaccines. Initially, edible plant vaccine research included evaluating the usefulness of plant-derived vaccine antigens against the enteric infections (Haq et al. 1995; Mason et al. 1996; Richter et al. 1996). Later, involvement of many vaccine research groups in this field of research resulted in the application of this technology in studying the effectiveness of plant-derived vaccine antigens both in experimental animals and to some extent in human models. Results published till date suggest the possibility of making Arntzen's inspired vision a reality of bringing transgenic plants, expressing vaccine antigens, to the clinics (Tacket et al. 1998, 2000; Thanavala et al. 2005). During the early years of edible vaccine research, vaccine antigens were mostly expressed in tobacco plants, as this has been the model plant in transgenic plant biotechnology. Many protein antigens, including of bacterial and viral pathogens, have been expressed, and their antigenicity and structural authenticity have been evaluated (Jani et al. 2004; Smith et al. 2003; Wang et al. 2001). Tobacco system has several advantages in terms of overall yield and the time required for running the experiments. Although this system carries some advantages, expression of edible vaccines for clinical evaluation in these plants is not recommended due to the presence of toxic alkaloids. Thus, any plant or specific parts of the plant that can be eaten raw are generally considered for the expression of vaccine antigens, although some proteins have been shown to retain antigenicity on cooking. With respect to edible vaccine development, the following criteria need to be considered before

selecting the plant expression host for transformation: (i) ease of transformation and the availability of standard protocols for specific plant species; (ii) production cost and maximum yields per acre land compared to other alternative methods for the production of the desired protein; (iii) total time required that includes generation of transgenics, testing the authenticity and efficacy of the expressed protein, preclinical and clinical studies, scale-up, and so on; (iv) the overall stability of the expressed proteins in the plant cell environments during storage and transportation; (v) a host that can be well contained so as to avoid the environmental hazards due to the spread of transgene into nontarget species; and (vi) the last and the most important which is the acceptability and suitability of the target tissue by different age groups, particularly children. Any host plant that satisfies the above criteria should be the best choice for the expression of recombinant vaccine antigens. Some of the crops suitable for vaccine production are discussed in the following sections.

13.3.6.1 Leafy Crops

Leafy crops are best suitable for animal vaccine production, although crops like lettuce have been used for the production of edible vaccines for human use. Tobacco was the first to be considered for the expression of recombinant proteins in leaves due to the long availability of standard protocols for its transformation, high biomass per acre land, prolific seed production, and the availability of well-established downstream processing technology. When edible vaccine production is considered, tobacco leaves are not edible, and thus, tobacco system can only be used for the production of therapeutic proteins, although the use of low-alkaloid varieties of tobacco may increase the acceptability of tobacco-derived vaccines (Kang et al. 2005). Other plants like nitrogen-fixing alfalfa and soybean can also be considered for leaf-based expression systems. Expression of foreign proteins in these plants has been well studied, and unlike other expression hosts, expression of recombinant proteins in alfalfa results in more homogenous glycosylation patterns. Considering the advantages of these leaf-based expression hosts, oral vaccines for animals can be effectively produced in these plants (Dus Santos et al. 2002, 2005; Perez Filgueira et al. 2004). Recently, HBV vaccine antigen produced in lettuce has been used in clinical trials, and this vaccine antigen successfully induced the immune system in human (Kapusta et al. 1999, 2001). However, the biggest disadvantage of expressing recombinant proteins in leaves is that the proteins are expressed in an aqueous environment, and, thus, their stability becomes a big concern, particularly if the transgenics need to be stored for longer time periods. Thus, expression of recombinant proteins, particularly edible vaccine antigens, in these vegetative organs is not always the best option, at least when there are other alternative hosts available.

13.3.6.2 Fruits and Vegetables

Fruits are readily consumed by children and adults alike; therefore, expression of edible vaccine antigens for human in the fruit is another good option. They can be ingested raw without any further processing. Even some of the vegetables, for example, carrot, can be taken raw or after partial cooking. These advantages make them the most suitable candidates for the production of recombinant edible vaccines. Several studies have reported the expression of vaccine antigens in potato and induction of immune responses against the potato-derived vaccine antigens both in animal models and in human clinical trials (Choi et al. 2005; Huang et al. 2005; Mechtcheriakova et al. 2006). The potatoes are little difficult to consume raw and sometimes results in dysentery and vomiting, at least in human beings. Due to these drawbacks, this system is not considered for large-scale production of human oral subunit vaccines. However, in a study, it has been shown that vaccine antigens expressed in potato tubers retain their immunogenicity to a great extent after boiling (Arakawa et al. 1998; Warzecha et al. 2003). This is encouraging, but the fact that levels of immunogenic antigens after boiling will be different and, thus, the dosage could be a problem. Tomatoes are also investigated for vaccine production, and these are more palatable than potatoes. They offer several advantages that include high biomass yields and that they can be ingested raw, even by children. Rabies antigen has been expressed in tomato and, when given to experimental animals, induced adequate immune responses (McGarvey et al. 1995). Another option is to express the vaccine antigens in fruits liked by young children. Banana has been considered as the expression host, and the biggest advantage of expressing in banana is that they are widely grown in the developing countries, where the vaccines are most needed (Fig. 13.3). It has been reported that promoter MaExp1 could be an important tool for expressing foreign proteins (vaccine) in banana fruit at the time of ripening (Nath 2004). Further research into fruit-based production of vaccines and therapeutics would increase their public acceptability, and also the certification from the health departments would become easier. Hence it needs special attention from research groups.

13.3.6.3 Cereals and Legumes

Expression of recombinant proteins in seeds offers several advantages including high stability, restrict horizontal transfer of transgenes to nontarget organisms, and so on. Seeds provide comparatively a dry medium for the expression of proteins, and, thus, unlike leaves and other vegetative organs, they provide a suitable biochemical environment for the stable expression and storage of recombinant proteins in a more concentrated form. Expressing the pharmaceutical proteins as fusion genes with the fusion partners, like oleosin, eases the purification using a simple protocol (developed by SemBiosys Genetics Inc.) followed by endo-protease digestion to separate the fusion partner to obtain the desired pharmaceutical protein in a pure form (Parmenter et al. 1995). Attempts have been made to produce viral and

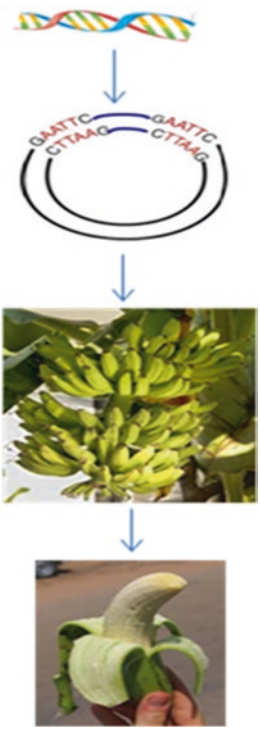

Fig. 13.3 Production of edible banana vaccine

bacterial vaccine antigens in seeds, and these studies clearly suggest the usefulness of cereals for the safe and stable production of recombinant vaccine antigens for human and animal use (Streatfield 2006; Lamphear et al. 2002, 2004). Here it should be noted that although not all cereals are readily palatable, they can be given after mild processing. It has been observed that even after long storage at room temperature, there was no considerable loss in the overall concentrations of active proteins in the seeds (Stoger et al. 2000). Even transportation becomes easy, as they don't need cold chain packaging while being transported, and thus can be transported at room temperatures. Although most of the cereals are not readily palatable, there are some exceptions like sprouts that can be eaten raw. On the other hand, it is too early to arrive at a conclusion that cooking makes these vaccine antigens immunologically inert, as the available information regarding this aspect is very much limited and, thus, needs proper evaluation in different expression hosts on a case-by-case basis before coming to a final conclusion about their feasibility as vaccine-production hosts. Targeted expression of recombinant proteins in seeds has biosafety advantages in that the nontargeted organisms are not affected by transgene products. However, there are several factors that limit the use of seeds for the production of therapeutic proteins. These include the following: (i) overall yields are much lower in seed crops, (ii) the plants need to go through a reproductive cycle to produce the seeds, and (iii) during their reproductive cycle, they can mediate gene flow through pollen transfer.

13.4 Plant-Derived Vaccines

In the present-day world, vaccination seems to be the safest option to protect ourselves from the existing and ever-growing pool of infectious organisms in the nature. Although a single vaccine that gives protection from multiple infectious organisms is most desirable, till date no such vaccine has reached the market for use, and, with the available technologies, it is highly impossible to produce vaccines that would suffice for mass vaccination of millions of population under threat. However, expression of vaccine antigens from different pathogens in plants seems to solve the problem of insufficiency and of transport to places, which do not have access to the currently available vaccines. Plant expression technology has gone one step ahead to produce multivalent vaccines offering the advantage of vaccinating against multiple pathogens in a single shot (Yu and Langridge 2001). Insights into the recombinant protein expression in plant systems revealed many similarities at different levels between plant and animal cell systems, and these studies also indicated the superiority of plant expression systems over the conventional expression systems in terms of safety, cost, and authenticity of the recombinant protein antigen. In the current scenario, we could achieve expression levels of recombinant protein up to 4 % of the total soluble protein (TSP) combined with the necessary posttranslational modifications through nuclear transformation and up to as high as 46 % of the TSP in transplastomic plants (Daniell et al. 2005; De Cosa et al. 2001; Kang et al. 2003; Koya et al. 2005). Till date, several vaccine antigens have been expressed in transgenic plants, and their antigenicity has been tested in experimental animals (Ashraf et al. 2005; Huang et al. 2005; Khandelwal et al. 2004; Koya et al. 2005; Mason et al. 1998) and some of them have even been taken to human clinical trials, where their ability to induce systemic immunity and mucosal immunity on oral administration is highly promising in the context of development of plant-derived edible vaccines for human and animal vaccination (Thanavala et al. 2005).

However, naïve proteins have been found to be poor immunogens after oral administration and antibody titers were found to be comparatively less even after repetitive oral administrations. This problem of inefficient immunogenicity can be overcome either by co-expressing the known mucosal adjuvants as fusion proteins or by simply mixing the mucosal adjuvants with the vaccine preparation before administration. When edible vaccines are considered, co-expression of mucosal adjuvant is most suitable. Several mucosal adjuvants have been proposed in the recent times and include either protein antigens or lipid-based molecules (Freytag and Clements 2005; Ogra et al. 2001). In fact, when talking about plant-derived vaccines, it should be noted that even plant DNAs and plant-derived products can act as strong inducers of immune system and, thus, can be used as adjuvants (Pickering et al. 2006; Wang et al. 2002). Plant DNAs containing hypermethylated CpG motifs in their genome, however, were found to be nonimmunogenic (Wang et al. 2002). Apart from the above, even viral proteins have been efficiently used to enhance the mucosal immune responses, wherein the gene of interest is co-expressed with a known potent inducer of viral origin. In most cases, these viral proteins are

multimeric self-assembling particles, and the desired protein is expressed on these particles (Brennan et al. 1999; Durrani et al. 1998; Wagner et al. 2004). Thus, depending on the expression vector and on the requirement for specific immune responses, the above options can be effectively used to enhance the mucosal immune responses against the desired gene product. Alternatively, the plant-derived vaccine antigens can be administered to boost the already existing immune responses from protein- or DNA-based immunizations. In this case, currently available vaccines or the DNA-based vaccines are given to prime the immune system, and the subsequent doses may consist of plant-derived vaccine antigens (Webster et al. 2002a). This approach is highly useful where the amount of plant-expressed protein is insufficient to prime the immune system and/or where multiple administrations of potential vaccines are expensive.

Vaccine candidates expressed in transgenic plant systems

Antigen/antibody protein	Production system	Efficacy/application	References
HRV-VP7 (human rotaviruses)	Potato	Elicited serum IgG and mucosal IgA by oral delivery to mice	Wu, Li et al. (2003)
IgG (hepatitis B virus)	Tobacco	Immunogenic to mice	Valdes, Gomez et al. (2003)
LT-B (heat-labile toxin B)	Maize kernels	Antibodies produced following feeding of potatoes to mice	Chikwamba, Scott et al. (2003)
TetC (tetanus vaccine antigen)	Tobacco chloroplast	Protective antibodies produced following intranasal immunization	Tregoning, Maliga et al. (2004)
CFA/I (colonization factor antigen/I)	Potato leaf and tuber tissues	Protective antibodies produced as incubated with Caco-2 human colon carcinoma cells	Lee, Yu et al. (2004)
PEDV-COE (core neutralizing epitope of *Porcine epidemic diarrhea virus*)	*Tobacco mosaic virus* to tobacco	Induced both systemic and mucosal immune responses by feeding to mice	Kang, (2004)
CTB-gp120 (HIV-1 gp 120 V3 cholera toxin B subunit fusion gene)	Potato	Induced significantly greater immune responses in mice	Kim, Gruber et al. (2004)
IgG (*Rabies virus*)	Tobacco	Systemic injection showing therapeutic activity	Ko, Wei et al. (2004)
HEV CP (HEV capsid proteins)	Potato leaf or tuber	Oral immunization of mice failed to elicit detectable antibody responses	Maloney, Takeda et al. (2005)
MV-H (measles virus hemagglutinin)	Tobacco	Indicative of humoral and mucosal immune responses by oral delivery	Webster et al. (2005)

(continued)

Antigen/antibody protein	Production system	Efficacy/application	References
HIV-1 Tat protein	*Tobacco mosaic virus* (TMV) to spinach	Immunogenic and protective by oral delivery to mice	Karasev, Foulke et al. (2005)
pBsVP6 human group A rotavirus	Alfalfa	Protecting from acute rotavirus-induced diarrhea by oral delivery to mice	Dong, Liang et al. (2005)
scFv-HER2 (human epidermal growth factor receptor-2)	Tobacco	Cancer diagnosis and therapy	Galeffi, Lombardi et al. (2005)
RSV (*Respiratory syncytial virus*)	*Nicotiana tabacum* cv. Samsun NN	Generating strong T cell responses in human dendritic cells	Yusibov et al. (2005)
HBsAg M protein	*N. benthamiana*	Antibody titers gradually increased by intraperitoneal injection to mice	Daniell, Streatfield et al. (2001)
NVCP (*Norwalk virus* capsid protein)	Tomato fruit of tomato fruit to mice	Immunogenic by oral delivery freeze-drying	Daniell, Chebolu et al. (2005)

13.5 Clinical Trials

Plants have been successfully used for antigen expression and production. There is, however, a need to preclude clinical trials in order to use the recombinant vaccines for the public benefits.

13.5.1 ETEC (Enterotoxigenic Escherichia coli*)*

First human trial of edible vaccines was accomplished in 1997 by Charles Arntzen at Boyce Thompson Institute, USA, for enterotoxigenic *Escherichia coli* (ETEC). The volunteers were fed raw transgenic potatoes expressing LT-B (ETEC). The 91 % of the volunteers were found to develop neutralizing antibodies, and 55 % developed mucosal response (Mason et al. 1998).

13.5.2 Cholera

Mice fed with transgenic potato (one potato a week for a month with periodic boosters) with CTB gene of *Vibrio cholera* provided immunity in mice (Arakawa et al. 1997). Nasal administration of a mutant cholera toxin subunit A (mCT-A) and LT-B co-expressed in crop seed has been shown to be effective (Yuki and Kiyono 2003).

13.5.3 Norwalk virus

Twenty volunteers were fed with transgenic potato expressing *Norwalk virus* antigen, and it was observed that 95 % of volunteers showed seroconversion (Tacket et al. 2000). Scientists are now focusing on the engineering of bananas and powdered tomatoes expressing *Norwalk virus*.

13.5.4 Measles

Measles virus hemagglutinin (MV-H) from Edmonston strain expressed in tobacco plant was fed to mice by Huang et al. (2001). The antibody titers found in mice were five times the level considered protective for humans. The mice were also shown to secrete IgA in their feces (Huang et al. 2001). Giving boosters by combining parenteral and subsequent oral MV-H doses could induce titers 20 times the human protective levels. The titers were found to be significantly greater than with either vaccine administered alone (Webster et al. 2002a, b).

13.5.5 Rabies

Tomato plants have been shown to express rabies antigens that could induce antibody production in mice (Prakash 1996). Transformed tomato plants using CaMV with the glycoprotein gene of *Rabies virus* (ERA strain) were shown to be immunogenic in animals (http://www.geocities.com/plantvaccines/futureprospects.htm.).

13.5.6 Hepatitis B

Parenteral virus-like particles (VLPs) could invoke specific antibodies in mice for hepatitis B (Thanavala et al. 1995). Potato-based vaccine against hepatitis B showed encouraging results when it was used for clinical trial purposes. It was found that a single potato could serve for the amount of HBsAg needed for one dose. An elevation in the levels of specific antibodies was observed that exceeded the protective level of 10 mIU/mL in humans. It was observed that a prime boost strategy in mice with a single sub-immunogenic parenteral dose of yeast-derived recombinant HBsAg and subsequent oral transgenic potatoes resulted in the development of antibodies that immediately elevated to > 1000 mIU/mL and were maintained at > 200 mIU/mL for 5 months (Richter et al. 2000). Nowadays guarded green houses have been designed to grow the tomatoes expressing hepatitis B.

13.5.7 HIV

Two HIV protein genes were successfully injected into tomatoes using CaMV as a promoter. The expressed protein was detected by polymerase chain reaction (PCR) in different parts of the plant, including the ripe fruit. It was also detected in second generation of the tomato plant (http://www.sciencedaily.com/releases/2004/08/040816001548.htm). Recently, spinach has been successfully transformed by TMV containing Tat protein. Leaf tissue of spinach was shown to contain up to 300–500 mg/g of Tat antigen (Karasev et al. 2005). The mice were fed with this spinach followed by DNA vaccinations showed higher antibody titer as compared to the controls, with the levels peaking at 4 weeks postvaccination.

13.5.8 Anthrax

Tobacco leaves were found to express a protein structurally identical to the major protein present in existing vaccine when bombarded with PAG gene (anthrax protective antigen – PA) using a gene gun. This vaccine was also found to be devoid of edema factor and lethal factor, responsible for the toxic side effects. This anthrax antigen has been now put in tomato plants. Due to public opposition to genetically modify tobacco plant for human consumption, spinach is seen as a safer plant. The attempts are on way to transform spinach by using TMV-expressing protective antigen (PA) (Sussman 2003).

13.6 Concerns

Plant biotechnology has made a revolution in the last two decades. Researchers exploited the interactions between the soil bacterium and the plants, which formed the basis for the idea of transgenic plant technology. Initially, this resulted in the production of high-quality varieties of crop plants, and then this has been utilized for the production of recombinant proteins of pharmaceutical and therapeutic use. All animals on this Earth, including human beings, consume plant-derived products in their daily diet, an idea that struck a scientist's mind some 15 years ago, and this resulted in the exploitation of transgenic plant biotechnology to produce the vaccine antigens. Lot of research has been dedicated to edible vaccine technology in the last 15 years, and this included analysis of structural authenticity and immunogenicity of many a number of plant-derived vaccine antigens for human and animal use (Dus Santos et al. 2002; Haq et al. 1995; Kang et al. 2003; Mason et al. 1996; Rossi et al. 2003). Efforts are also underway to understand the mechanisms involved in the expression of recombinant proteins in transgenic plants. Many therapeutic genes had been expressed in transgenic plants, and their immunogenic potency was analyzed in experimental animal models. Few of them have been considered for clinical

trials and the results are highly promising. However, the knowledge we have gained from these experiments is comparatively very less, and lot of work needs to be done before drawing conclusions regarding their widespread use in clinics. In the current scenario, there are many concerns pertaining to the effect of these GMOs on the nature and their safety, when given to human beings, in the long run. In this context, the current understanding is based mostly on the natural events occurring both between different plant species and also between plants and other species. Before we actually accept the plant-derived vaccine and pharmaceutical proteins for routine use, it's highly necessary that we acquire more knowledge about their possible hazards, both on the nature and on the animal species. These factors are discussed in the following text with some examples from the available literature.

The biggest concern is the possible deleterious effects due to introgression, a process involving transfer and integration of genes from one population to another, of transgenes from transgenic plants forming new combinations that never existed in the nature. The consequences could be the generation of new and more diverse viruses, antibiotic resistant organisms, generation of herbicide-resistant weeds, and those with abnormal levels of expression of their metabolites, thereby disturbing the ecological balance in the nature. Although introgression is not as simple as it is described and takes many generations to stably exist in nature, this itself is a major constraint in studying precise introgression events between GMOs and normal species. There are experimental evidences combined with data on natural events, between related plant species, suggesting the existence of introgression in the nature (Ellstrand and Schierenbeck 2000; Quist and Chapela 2001; Zhou et al. 1997). A group of scientists, those who are against the very idea of transgenic plant biotechnology, feels that using constitutive promoters, like CaMV, would lead to unintentional consequences due to random recombination. Their argument is based on some natural introgression events and recent findings showing the occurrence of transgenes, which are basic components of most of the T-DNA cassettes in the plant expression vectors, among the wild plants that are growing in close proximity to the transgenic crops(Kwit et al. 2011; Lu 2013) (Cummins et al. 2000; Wintermantel and Schoelz 1996).

Natural introgression has been reported between diverse families with different characteristics, and the number of cases reported is far from the actual figure. Introgression events that occur between closely related species could not be studied due to the lack of specific molecular and morphological markers, and there is also a bias against detecting the recent introgression events, as they could be lost over a period of time (Quist and Chapela 2001; Rieseberg and Wendel 1993; Rieseberg et al. 2000; Stewart et al. 2003). Therefore, the number of confirmed cases reported till date represents only a minimum of the actual number, and the information available from these studies is also insufficient to extrapolate for the analysis of such events taking place between the GMOs under study and their wild relatives. Particularly, in case of transgenic plants, it is important to study the gene transfers between the GM plants and their closely related wild species to avoid the invasion, when they are introduced into the fields, of aggressive transgenic weedy plants into nature. The use of molecular markers, based on DNA polymorphisms, has greatly

assisted in understanding the interspecific introgression events (Rieseberg et al. 2000). Introgression has been shown to occur between different species that are geographically separated (Barton and Hewitt 1985). These facts, in combination with the experimental data, about the natural gene transfer events highlight the danger of introgression of transgenes from GM crops to their wild types.

However, the mechanism of gene transfer between the related species doesn't seem to be random, and genetic barriers seem to play a role in controlling the gene flow. In a study, on the introgression events between two *Helianthus* species (*Helianthus annuus* and *Helianthus petiolaris*), it was observed that gene transfer takes place only when the regions of the genome of the recipient species had the same chromosomal arrangement as the donor species, indicating that chromosomal rearrangements act as an important barriers to introgression (Rieseberg et al. 1995, 1999). Therefore, it appears that only the positively selected alleles will introgress, whereas the negatively selected alleles are retarded. A similar mechanism is predicted for the horizontal gene transfer between transgenic plants and their wild relatives. Complete understanding of these chromosomal blocks and their locations in the genomes of plant expression hosts might help in selecting those transgenics, in which the transgenes are linked to weak loci that are retarded when transferred to a nontarget plant. There are several other factors that influence the introgression of transgenes, and this illustrates that gene transfer is a more complex process. Different crops, including those used for transgenic plant production, have been divided into categories depending on the risk of gene flow from the agronomically important plants, in particular GMOs, to their wild relatives, and those that fall into very low-risk and low-risk groups are recommended for transgenic plant production, albeit with necessary precautionary measures (Stewart et al. 2003). The risk of transgene flow into nontarget organisms depends mainly on the potential ecological effect of the transgene on the recipient wild plant.

Chloroplast engineering of transgenes has been proposed as a barrier to transgene introgression, as chloroplasts are maternally inherited. This strategy will only be effective as long as the transgenic plant doesn't serve as the female partner in hybridization and further backcrosses, which is not always the case (Corriveau and Coleman 1998; Reboud and Zeyl 1994). Other strategies include the use of reversible suppressor genes under the control of seed-specific or pollen-specific promoters to produce sterile plants. In these cases, the effect of suppressor genes can be reverted when required by crossing with those expressing anti-suppressor genes. However, when transferred to nontarget plants, the suppressor genes would be active and would never allow persistence of these plants in the nature. Strategies involving the production of sterile male plants have been successful, whereas those involving the production of sterile seeds have created controversies and, thus, need further investigation before implementation. With respect to transgenic plants, carrying genes for animal and human vaccine antigens, it is highly unlikely that they confer any positive fitness effect to the recipient plants and, thus, these plants can be grown in large scale in open fields with minimal levels of containment measures. However, the transfer of antibiotic resistance genes to bacteria is of some concern, although no experimental data till date suggest the occurrence of any such gene

transfer to surrounding microflora. Another important concern is with respect to the effect of GM crops on human health. It was predicted that when GM crops are fed to humans, the antibiotic resistance marker used for selection of transgenic plants might be transferred to the intestinal bacteria and then to the pathogenic bacteria. If this occurs, this will have a serious negative effect on human health as these pathogens would acquire resistance against some of the commonly used antibiotics. Thus, it is highly desirable, before any final decision is taken to introduce GMOs into market, to assess the risk of marker gene transfer to pathogenic bacteria through stringent experimental procedures.

Apart from the above problems, production of plant-derived edible vaccines and pharmaceutical proteins for human use is also raising safety concerns. As mentioned above, plant-derived heterologous proteins possess some plant-specific glycans. These plant glycans are found to be the targets for IgE binding and, thus, are suspected to cause allergic immune reactions in animals (van Ree et al. 2000). These predictions arise from the earlier experiences with the therapeutic antibodies in use that are produced in mammalian cells, where some of the patients administered with these antibodies developed immune responses against the substrate cell-specific glycans present on them. Yet, there is no strong evidence in the literature supporting the view that these plant-specific glycans are immunogenic when a plant-derived vaccine antigen is administered orally. This is not entirely surprising, as our daily diet includes lots of plant-derived products and, still, it doesn't result in any adverse response. Taking this into consideration and the mechanism of oral tolerance, it could be possible that all the animals, including human beings, must have developed tolerance to these plant glycans. Although there are some reports of immunogenicity against the plant-derived glyco-epitopes, they are derived from the studies on those, who are allergic to plant glyco-epitopes or on specific animal species immunized by other than oral route (Bardor et al. 2003). The clinical significance of these anti-glycan immune responses is uncertain, and this needs further investigation. Even if there is any immune response against the plant-specific glycans present on the injected vaccine antigen, it has strong positive effect on the efficiency of the given oral vaccine antigen, as they help in attracting the antigen presenting cells toward the vaccine antigen.

13.7 Conclusions

Plant expression of proteins of diverse use, particularly the vaccine antigens, is produced and analyzed in the laboratory. Some of them have even reached the human clinical trials and proved the efficacy and usefulness of plants as production hosts for edible vaccines. However, plant expression of heterologous proteins is not a straightforward process, and once a protein is expressed and its efficacy tested in the laboratory, there are many aspects that need to be considered before planning for large-scale production followed by use in clinics, including the regulatory issues on plant-derived products. There are several instances when researchers have failed to

express heterologous proteins in transgenic plants even at the laboratory level largely due to issues related to expression vector design. By considering the criteria discussed above, one should be able to efficiently carryout the heterologous protein expression in plant system. However, more insights into the heterologous gene regulation and expression patterns in transgenic plants could answer some of the unknown mechanisms that cause heterologous gene expression a tough task. Although there are several reports related to silencing of transgene expression in plants, very little is known about any such mechanisms involved in the silencing of vaccine antigen expression in transgenic plants. More insights into the possible cause for the shutdown of transgene expression in these plants carrying the genes coding for vaccine antigens would be more useful. Apart from these issues, concerns related to the release of transgenic plants into the market and their widespread use need to be investigated thoroughly to avoid any unwarranted issues in the long run. With respect to plants used for the production of edible vaccines for human and animal use, although the environmental issues are less important considering the fact that these genes coding for vaccine antigens do not offer any advantage to the nontarget plants when introgressed, problems associated with the toxicity of these products in humans need to be sorted out before they are introduced into the clinics.

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Chapter 14
Biosafety, Bioethics, and IPR Issues in Plant Biotechnology

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Abstract Plant biotechnology holds great promises for feeding the world's growing population and for improving the diets of people the world over. To feed the ever-increasing population, global agricultural production may need to be increased by 60–110 % by 2050. First-generation transgenic plants focused on increased yield and other agronomic properties, which primarily benefited agribusiness companies and farmers, whereas the second generation of genetically modified foods emphasized on consumer health. Although concerns over ecological and human health safety have led to doubt over the application of transgenic technology, many of these fears seem unsubstantiated or are based on misinformation.

To evaluate the applicability of genetically modified food and development of new product for the public benefit, efficient collaboration between the field of plant science and nutrition is required. With plant biotechnology promising to deliver great benefits for both producer and consumers, its application is also associated with the potential fear about the concentration of economic power, infringement of developed technologies, and technological dependence, which could deepen the technological gap between developed and developing countries. Appropriate policies developed and publicized can ensure to accurately check the misuse and avoid unnecessary inconvenience.

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14.1 Introduction

The exponential increase in the world population and decrease in crop productivity had dimmed the achievements of green revolution. With the continual expanding global population, the world economy is facing challenges of malnutrition, hunger, and poverty. To meet the nutritional needs, without bringing new farmland into production, it is estimated that yields of rice, wheat, soybean, and maize all need to be doubled by 2050 (Ray et al. 2013). The decline in the crop productivity was due to the drastic changes in climate across the globe, imbalanced nutrients in the soil, altered soil texture, disturbed soil microflora and fauna, frequent incidence of diseases, insect/pest attacks, and exposure of crops to abiotic stresses during developmental stages (Godfray et al. 2010). At this juncture, when there is large gap between demand and supply of the food, the productivity of these crops should be improved by developing novel crop varieties having traits that help them to efficiently extract and utilize water and nutrients from the soil, tolerate/resist biotic and abiotic stresses, translocate photosynthates efficiently to the economic sink, and have a compact plant type that may ensure higher plant density. This will ensure food security which is endangered in the present scenario.

Plant transgenic technology had enabled plant scientists to develop more useful and productive crop varieties by bringing genes encoding specific characters from a wide range of live sources (Young 2004). Using transgenic technology, depending on variety, place, time, and purpose of the plant grown, desirable gene(s) can be incorporated to express beneficial novel trait(s) in desired crop. Thus, transgenic plants with insect resistance, herbicide tolerance, disease resistance, high nutritional quality and high yield potential, delayed fruit ripening, and enhanced ornamental value are now developed and grown globally. Genetically modified (GM) plants are also used as bioreactors for the production of biopolymers, nutraceuticals, antigens, vaccines, and monoclonal antibody.

Upsurge in biotechnological products and services has attracted big players worldwide. With strong scientific and technical pool of human resources, rich biodiversity, and a large agricultural base, India has been recognized as a producer of low-cost, high-quality bulk drugs and formulations and other biotech products and services. As the Indian market harbors some of the top biotech firms and companies functioning in India, it becomes imperative to make effective protection regime for the prevention of any kind of misuse and abuse in this sector.

Agri-Biotech as a subsector is among the fastest-growing biotech sectors in India. It has witnessed the emergence of a single large dominant enterprise, namely, Monsanto that has been alleged by competitors and consumers alike of abusing its dominant position and engaging in behaviors and activities that are prima facie anticompetitive and against the interests of consumers. In India as well as global context, this assumes critical importance with the growing concerns relating to food security.

This chapter identifies valid concerns and risks, recounts regulatory and adoption practices developing transgenic crop and effective protection regime for generated IPR, and provides useful information to lessen the unsubstantiated fears based on misinformation.

14.2 Status of Transgenic Crops

With the discovery of technologies for gene transfer from one organism to another, incorporation of beneficial traits into plants became possible. The transgenic plants were developed and applied successfully first under contained conditions for experiments (Cramer 1996) followed by infield and open-environment condition for commercial applications.

The adoption rate of transgenic crops, however, shows great disparities between different agricultural producing regions worldwide. Farmers especially in developing countries are greatly benefited by increased yields of transgenic crops. The adoption of transgenic crops can further reduce the production costs by reducing pesticide and labor use. A recent meta-analysis concluded that transgenic technology has drastically reduced the use of pesticide by 37 %, increased crop yields by 22 %, and increased farmer profits by 68 % (Klümper and Qaim 2014).

The news of genetically modified FLAVR SAVR tomato with delayed premature fruit softening was received with dramatic responses. The hype against adoption of GM crop technologies was fueled by public misperceptions and prejudice about eating "mutant vegetables." After extensive safety research, Calgene's tomato was found to be safe. In 1994, FDA finally approved the commercial release of FLAVR SAVR tomato. After this there was a rapid increase in GM crop cultivation. Insect-resistant Bt maize as well as Bt cotton and transgenic herbicide-tolerant soybeans as well as oilseed rape were planted on rapidly increasing areas. In 2014, genetically modified (GM) crops were grown by 18 million farmers in 28 countries on a total area of 181.5 million hectares, which correspond to already 13 % of the world's arable land. This rapid increase makes biotech crops the fastest adopted crop technology of the last decade (Clive 2014; Lucht 2015) (Table 14.1).

The annual global hectarage of biotech crops was 179.7 million hectares in 2015 as compared to 181.5 million in 2014. This change is equivalent to a net marginal-to-year change of minus 1 % between 2014 and 2015. Increase and decrease in annual hectarage of biotech crop are influenced by factors such as decreased overall crop plantings (for cotton it was minus 5 % and maize minus 4 %), low procurement prices, and farmers switching from cotton, maize, and canola to a more easily managed crop such as transgenic soybean. However, there is an overall decrease in biotech crop hectarage which was driven by low prices of plant produce in 2015 (Clive 2015).

GM technologies resulting in commercially produced varieties in countries such as the USA, Canada, and European Union have centered on conferring resistance against bacteria, viruses, or insect pests and producing specific herbicide-tolerant plants. The first transgenic plant accepted and approved for use in 1998 in the EU was Corn MON 810, a variety developed by the Monsanto Company, USA. It contained cry gene (commonly called Bt gene) from *Bacillus thuringiensis*

Table 14.1 Global area of biotech crops in 2015, by country (million hectares)[a]

Rank	Country	Area (million hectares)	Biotech crops
1	USA[b]	70.9	Maize, soybean, cotton, canola, sugar beet, alfalfa, papaya, squash
2	Brazil[b]	44.2	Soybean, maize, cotton
3	Argentina[b]	24.5	Soybean, maize, cotton
4	India[b]	11.0	Cotton
5	Canada[b]	11.6	Canola, maize, soybean, sugar beet
6	China[b]	3.7	Cotton, papaya, poplar
7	Paraguay[b]	3.6	Soybean, maize, cotton
8	Pakistan[b]	2.9	Cotton
9	South Africa [b]	2.3	Maize, soybean, cotton
10	Uruguay[b]	1.4	Soybean, maize
11	Bolivia[b]	1.1	Soybean
12	Philippines[b]	0.7	Maize
13	Australia[b]	0.7	Cotton, canola
14	Burkina Faso[b]	0.4	Cotton
15	Myanmar[b]	0.3	Cotton
16	Mexico[b]	0.2	Cotton, soybean
17	Spain[b]	0.1	Maize
18	Colombia[b]	0.1	Cotton, maize
19	Sudan[b]	0.1	Cotton
20	Honduras	<0.1	Maize
21	Chile	<0.1	Maize, soybean, canola
22	Portugal	<0.1	Maize
23	Cuba	<0.1	Maize
24	Czech Republic	<0.1	Maize
25	Slovakia	<0.1	Maize
26	Costa Rica	<0.1	Cotton, soybean
27	Bangladesh	<0.1	Brinjal/eggplant
28	Romania	<0.1	Maize
	Total	179.7	

Source: Clive James (2015)

[a]Rounded off to the nearest hundred thousand

[b]19 biotech mega-countries growing 50,000 ha, or more, of biotech crops

that produces proteinaceous toxin inside the bacterial cells. It kills the insect pests specifically coleopterans, lepidopteran, and dipteran. Bt transgenic crops (rice, eggplant, brinjal, cabbage, cauliflower, canola, cotton, corn, maize, tobacco, potato, tomato, soybean, etc.) have been developed and grown in the USA, Brazil, India, China, Argentina, South Africa, etc. Amflora potato became the second GM transgenic plant to be accepted by the European Commission for production in EU

in 2010 (Devos et al., 2006) after long registration period (12 years). The BASF (Ludwigshafen, Germany) succeeded in developing EH92-527-1 (common name Amflora) variety by suppressing the genes for the production of amylase. This variety produces over 98 % of amylopectin (Clive 2010) and has advantage in industry over the normal potato variety as the two starch types don't need to be separated. Another important trait incorporated in plants is herbicide tolerance. Herbicide-tolerant (Ht) crops are genetically modified to survive particular herbicide applications used to check the weed growth. The "Roundup Ready" crop, which is genetically modified by Monsanto to tolerate applications of the company's glyphosate-based herbicide, "Roundup," is the most common Ht crops today.

Four GM crops (corn, soy, cotton, and canola) account for 99 % of acreage under GM cultivation around the globe. GM corn, soy, and canola (and cottonseed oil) proliferate in our food system as ingredients in processed food and in animal feed. GM soybean is cultivated in nearly half of the total GM hectares in the world followed by GM corn (30 %), GM cotton (14 %), and GM canola (5 %). The technology is still to realize its potential in GM crops (fruits and vegetables or GM grains) that are consumed as whole foods. GM squash varieties (USA), GM papaya (USA and China), GM eggplant (Bangladesh), GM sugar beet (Canada and the USA), and GM alfalfa (USA) collectively account for only 1 % of the global GM crop hectares. In 2014, GM herbicide tolerance comprised of 57 % of the world's transgenic crops, 15 % were GM plants toxic to pests, and 28 % were GM plants for insect resistance and herbicide tolerance. Further, GM crops for drought tolerance and virus resistance together account for less than 1 % of global transgenic hectares. The five fast developing countries in transgenic crop production and commercialization are China and India in Asia, Argentina and Brazil in Latin America, and South Africa (Clive 2014). All these collectively grew 71.4 million hectares of the GM crops (44 % of global acreages).

In 2014, 29 countries (among which 79 % were developing countries) approved commercial cultivation of transgenic crops, 31 countries (among which 70 % were developing countries) for food, and 19 countries (among which 80 % developing countries) for feed. The highest number of country approvals of GM crops for feed was in 2011, whereas highest number of country approvals of GM crops for food was in 2014. The data suggest the increasing acceptance of the technology for human use. Highest approved GM crops were for herbicide tolerance followed by insect tolerance. Maize had the highest number of events approved (single as well as stacked traits). Stacking of traits in GM crops is gradually increasing and constitutes approximately 30 % of the total trait approvals. These results indicate growing understanding and acceptance by various countries to be able to effectively exploit commercialization of GM crop (Aldemita et al. 2015).

Around 50 different kinds of genetically engineered plants, each developed from a unique event, have been approved for commercial cultivation in the USA. Table 14.2 enlists some of the commercial traits modified in different crops.

Table 14.2 Crops with various commercial traits

Crops	Modified trait
Soybean, maize, sugarcane	Abiotic stress tolerance
Soybean, maize	Altered growth/yield
Corn, soybean, cotton, canola, sugar beet, rice, flax	Herbicide tolerance
Papaya, squash, potato	Virus resistance
Canola, soybean	Altered oil composition
Tomato	Delayed fruit ripening
Chicory, corn	Male sterility and restorer system (used to facilitate plant breeding)
Corn, cotton, potato, tomato	Insect resistance

14.3 Transgenic Plant in India

The green revolution ushered in the late 1960s has led to a dramatic increase in crop productivity due to introduction of high-yielding seed varieties and the use of fertilizer and irrigation. With farm holdings shrinking in many parts of India due to urbanization, agricultural sector in India is under stress of feeding 1.5 billion mouths more than China by 2030; the challenge is now to replicate this success again. Another such revolution is required in edible oil and vegetable production demand for which is increasing day by day. Edible oil has become the third-biggest imported item by India, after crude oil and gold. India spends $10 billion annually to import edible oil which make for the 60 % of required edible oil.

Bt cotton is resistant to *lepidopteran* insect pests, which incur huge economic losses due to crop failure ranging between 80 % and 100 %, especially in Indian subcontinent. Bt cotton was the first GM crop to be introduced in India in 2002. Even after 12 years of the first GM crop (Bt cotton, a nonfood crop) planted in India, the country is yet to approve the commercial cultivation of GM crops for animal and human consumption. In fact, India is still pondering to allowing field experiments for GM food crops and very recently lifted a year-long embargo on scientific field trials. Bt cotton covers around 95 % of the total cotton growing area. In a short span of 12 years, around 7.7 million farmers have adopted Bt cotton in India (Clive 2015). Further 1128 varieties of Bt cotton transgenic plants have been allowed to be commercially cultivated in various zones of India, by GEAC from 2002 to 2012. The cultivation of Bt cotton has remarkably reduced insecticide used against these pests by 95 %. Further, India's total contribution in world cotton market has risen from 14 % (2002–2003) to 25 % (2014).

Similar to Bt cotton, the release of Bt brinjal was proposed by GEAC; however, its release was stalled in 2010 due to opposition. Bangladesh, in 2013, joined the list of Bt brinjal cultivation and is currently grown in about 50,000 ha. China is the sixth-largest adopter of GM crops worldwide after the USA, Brazil, Argentina, India, and Canada. It grows GM crops (papaya, cotton, poplar, sweet pepper, and tomato)

on over 4 mha. Among other Asian countries, the Philippines grows GM maize covering 62 % of the total area under maize cultivation (Clive 2014).

In India, approximately 18 major crops at present are at various stages of development and/or field trials in India. These include cabbage, brinjal, castor, chickpea, cauliflower, corn, groundnut, cotton, mustard, papaya, okra, potato, rubber, rice, tomato, watermelon, sugarcane, and sorghum. These crops have been targeted for resistance to viral and fungal diseases, insect pests, tolerance to pesticides, male sterility, nutritional enhancement, and tolerance to salinity and drought. There are around 12 government universities and institutes, and not less than 16 private sector organizations, contributing to this research, in India.

14.4 Public Fears

From the beginning of transgenic crop introduction, GM foods benefited the agricultural production, ecosystem, and human health. Industries, governments, and many academic scientists advocated the benefits of GM foods during the 1990s, by actively performing experiments and publishing research on plant transformation and biosafety. This resulted in adoption of GM varieties by farmers at record speed. This pace, however, was nudged by a research finding in 1998 showing GM potatoes expressing a gene for snowdrop sugar-binding protein showed stunted growth in rats and compromised their immune systems (Ewen and Putztai et al. 1999; Enserink 1999). This focused media attention on GM crops and fueled a rumpus in Europe against GM crops and food. With pollen from BT corn found to be toxic to the monarch butterfly, public concern aggravated (Losey et al., 1999). The monarch butterfly study showed that the danger to nontarget insects had not been thoroughly taken into account prior to commercial release. Subsequently, in various public forums, consumers, religious organizations, environmental activists, and some scientists warn of unforeseen human and animal health, environment, and socioeconomic consequences. All this had led to mistrust in the transgenic technology GM foods that gave rise to the concerns about the following issues.

14.4.1 Damage to Mankind

14.4.1.1 Allergenicity

The transgenic technology made possible the biofortification of food with vitamins and essential metals to enhance their nutritional value. At the same time however, the development of transgenic plants (especially staple food crops) with improved nutritional values has also come under the clouds of apprehension that they may introduce allergens into the food supply. So far there is no concrete evidence that gene products that are not allergenic normally will become allergenic when

expressed in a transgenic plant. As no reports on allergies to plant ferritin existed, it was, therefore, concluded that transgenic rice with the entire coding sequence of the soybean ferritin gene poses no allergenicity risk (Goto et al. 1999). The nature of genes that are selected for introduction into engineered host plant, however may some time induce allergic reactions. The reports of allergenic reaction to transgenic soybean in 1996, however, created a fear about GM food among the common people. Brazil nut albumin was expressed in soybean to improve the nutritional quality. The transgenic soybean expressed high levels of methionine which otherwise is low in the plant. The serum from Brazil nut allergic subjects, which were nonallergic to native soybean, were found to react with the transgenic soybean (Nordlee et al. 1996). It was hence concluded that the gene chosen to improve nutritional quality of soybean was one of the genes that trigger allergic reactions. Consequently, this line of soybean was not commercialized as a safety precaution.

Allergenicity assessment is complicated, when the allergenicity of a transgenic protein is not known. Over 200 food allergens have been identified and sequenced; however, no common motif or consensus sequence has been discovered (Gendel 1998). Physicochemical properties of target protein should be examined for potential allergenicity (Mendieta et al. 1997). Stability of a protein during the digestive process is another way to identify potential allergens, as most of the known food allergens are resistant to digestive acids and enzymes (Astwood et al. 1996). If a protein is degraded in the stomach and small intestine, then it is unlikely to reach immune cells and elicit a response. Further, the DNA sequence and structural similarity of the transgenic protein to known allergens should also be examined. Novel proteins that are stable inside the body can further be tested for reactivity with serum from subjects allergic to the homologous allergen (Ladics and Selgrade, 2009). Allergens are common in regular food, but over 90 % of the people having food allergies are allergic to one or more of the following foods: wheat, legumes, nuts, seafoods, eggs, or cow's milk. Thus, even in the absence of exhaustive methods for identifying potential allergenicity, the vast majority of transgenic proteins is suggested to be safe for consumption (Lehrer et al. 1996).

14.4.1.2 Antibiotic Resistance

For determination of the successful genetically modified plant, antibiotic resistance markers are being used. These markers have been selected by scientists after performing extensive safety studies and confer resistance to a narrow range of specific antibiotics with limited application in veterinary and human medicines. The rampant development of the resistance to existing antibiotics, however, has given rise to considerable public concern for the use of antibiotic resistance markers in transgenic crops. As DNA does not always fully defragment in the digestive system, human gut microflora and pathogens can take up modified genetic materials including gene for antibiotic resistance. This horizontal transfer may lead to reduction of effectiveness of antimicrobial therapy (Chen et al. 2010). This fear is termed as unwarranted by scientists with reasons as follows: (1) the antibiotic genes occur

frequently and naturally in soil and gut microbes without any human intervention, and (2) the transfer of genes from a plant to a bacterium is extremely unlikely. Thus, the proponents of commercial production of GM foods believe that these foods are safe as no evidence so far suggested the transfer of transgene from the GM food to gut bacteria.

The most widely used antibiotic resistance marker for the selection of transformed plant cells is the nptII gene, which confers resistance to the antibiotics neomycin and kanamycin. Kanamycin- and neomycin-resistant bacteria are ubiquitous in nature. On an average, 20–40 % of the bacteria that occur naturally in animal or human digestive tracts are already resistant to kanamycin. Moreover, these antibiotics have very limited application in humans and veterinary medicines. This gene is, therefore, most widely used as selection marker to develop GM crops with delayed fruit ripening and herbicide tolerance.

Although the risks from antibiotic resistance genes in transgenic plants appear to be low, still precautions can be taken to make it even more unlikely and resolve the public issues. Scientists can avoid using gene and protein sequences with homology to known bacterial genomes and proteomes, for the development of transgenic crops. Antibiotics that are not commonly used for the treatment of human diseases can be used to avoid acquired resistance to the antibiotics in case of any horizontal gene transfer to microorganisms in the body. Marker genes can be bred out leading to removal of the antibiotic resistance genes before the plants are released for commercial use, so that these genes can only be used during development and finally eliminated from the end product (Dale and Ow, 1991; Zuo et al. 2001; Rukavtsova et al. 2013; Ebinuma et al. 2001). Other non-antibiotic markers like mannose, GFP, or GUS can be used (Joersbo et al. 1998).

14.4.1.3 Eating Foreign DNA

Another public fear against GM food is that normal food could become metabolically dangerous or even toxic because of the presence of DNA fragment that is not naturally present in that plant. Very often these pieces of DNA come from either related (trans) plants or entirely different species (cis) such as viruses and bacteria. The properties of transgenes may change in a new chemical environment. Even if transgene itself is not dangerous or toxic, the presence could upset complex metabolic networks leading to production of new compounds or change the concentration of unseen and unwanted compound (Bawa and Anilakumar 2013).

We take in DNA unintentionally in abundance, in the form of food products, bacteria, and viruses found clinging to the meats, cereals, and vegetables. But there have not been any conclusive reports that DNA from transgenic crops is more hazardous to us than DNA from the conventional crops, animals, and microorganisms infesting them which we have been eating all through our lives. Schubbert et al. (1997) detected about 5 % of the DNA, consisting of short pieces (100 base pairs to 1700 base pairs long) in the small intestine, large intestine, and feces of mice fed with harmless detectable DNA sequence, up to 8 hours after a meal. A very small

amount of DNA (about 0.05 %) was found in the blood stream up to 8 hours after feeding. A study tracking the survival of the transgene *epsps* from GM soybean, in the small intestine of human ileostomists, showed low-frequency gene transfer from GM soybean to the microflora of the small intestine (Netherwood et al. 2004). The low level of *epsps* in the intestinal microflora even after consumption of the GM soybean meal showed that gene transfer did not occur during the feeding experiment. Thus, the possibility of transgene transfer from GM plants to intestinal enterocytes and microflora is unlikely (Netherwood et al. 2004).

14.4.1.4 Vector and Vector Elements

To make a GM crop, the gene of interest is mobilized and integrated into the target crop's genome using a DNA delivery vector. This vector might contain important elements, including viral promoters, marker genes, antibiotic resistance, and transcription terminators. The transgenes incorporated may reside anywhere in the genome, causing mutation in the host genome, and move or rearrange after its insertion in the same or next generations. It might break up and cause recombination (reintegrate into the genome) leading to chromosomal rearrangement in successive generations. This recombination could potentially change the GM plant such that it might produce protein(s) that causes specific allergy or related health problems (Goto et al. 1999; Hammond 1996).

Keeping the safety of animals and humans in mind, after extensive safety studies, scientists have proposed some promoters which are safe to use. Constitutive 35S promoter of cauliflower mosaic virus (CaMV) is the most widely used promoter in transgenic technology. CaMV causes cauliflower mosaic disease in broccoli, cauliflower, canola, and cabbage. Although other promoters have been used in developing transgenic crops, however, the CaMV promoter remains the first choice in laboratory as it drives large amount of protein production in a wide variety of situations. The opponents of the technology fear that the fragments of DNA may integrate into the host genome and cause ill effects. However, till now there is no evidence of invasion of CaMV promoter in human genome and function as a promoter regulating the unwanted nonfunctioning gene(s). Human chromosomes do contain the bits and pieces of DNA sequences from many different origins; however, these sequences have become nonfunctional due to multiple internals that have occurred during the course of evolution, over thousands of years.

14.4.1.5 Changed Nutrient Levels

Constant concern of the public against the use of transgenic technology is about the nutritional equivalence of genetically modified foods to their naturally occurring counterparts. Reduced levels of isoflavones (genistin and daidzin) were found in Roundup Ready® soybeans and thus having positive effect on human health

(Lappé et al. 1999). These two compounds have potential to prevent heart disease, breast cancer, and osteoporosis. Study performed by Monsanto suggested that Roundup Ready soybeans and unmodified soybean contain nearly the same amounts of isoflavones (Padgette et al. 1996; Clark and Ipharraguerre 2001). A conclusive study has not been performed; however, a comparison of available results reveals only small differences. Further, variability in isoflavone content is common in soybeans due to individual varietal influences and environmental factors such as weather, soil nutrient, etc. Also, samples of both Roundup Ready soybeans and the conventional counterparts produced similar growth and feed efficiencies for chickens, quail, rats, and catfish (Hammond et al. 1996; Clark and Ipharraguerre 2001).

14.4.2 Damage to the Natural Environment

The opponents of the technology have expressed great concerns about GM plants with regard to their adverse effect on the wildlife populations, environment, biodiversity, and wild relatives. The escape of transgenes and potential integration and establishment into the natural or agricultural ecosystems is threat to developing GM crops.

14.4.2.1 Impact on Nontarget Organisms

The impact on the nontarget organisms is a significant concern expressed by the opponents of GM foods. Pest-resistant crops may produce toxin which may have detrimental effect on other organisms including animals, plants, and microorganisms and is an important public concern regarding transgenic crops. Saxena and Stotzky (2001) showed that Bt toxin released from biomass of Bt corn and root exudates has no obvious effect on soil protozoa, bacteria, fungi, nematodes, and earthworms. The studies, however, showed that insect-resistant transgenic crops may negatively affect the beneficial insect predators such as ladybird beetles (Birch et al. 1997) and lacewings (Hilbeck et al. 1998).

With decrease in monarch butterfly population, in and around the fields of GM corn containing Cry1 toxins, public concern grew very strong. Studies showed that the butterfly population was reduced by destruction of their habitat (milkweed) by killer herbicides as well as by the ingestion of deposited pollens of Bt corn on milkweed leaves by butterfly. High mortality was observed in monarch butterfly larvae feeding on milkweed leaves dusted with Bt corn pollen, whereas no mortality in larvae feeding on leaves dusted with pollen from non-transgenic corn (Losey et al. 1999). Another study, however, showed negligible impact of pollens from Bt corn on monarch butterfly (Sears et al. 2001; Wraight et al. 2000). All the studies were based on laboratory experiments; therefore, the exact threat to monarch butterfly population in field conditions is difficult to assess.

14.4.2.2 Super Plants

The suspicion among critics of the technology aroused because of the fact that interbreeding between cultivated plants and their wild relatives is a natural and constant phenomenon and the transgenic plants are no exception (Snow 2002). Cross-pollination of crops with nearby related weeds may enable weeds to acquire newer traits and pose real threat to environment by giving rise to super plants. Many cultivated crops are sexually compatible with their wild relatives. They could, therefore, hybridize with them under favorable circumstances. In case of GM crop hybridizing with its wild relatives, the wild plant might take up transgenes. This introgression may change their behavior in a way that they could be a serious threat as competitors or weeds in natural environment. However, the likelihood that transgenes spread in particular part of the world can be different for each crop. For example, cross-pollination of transgenic corn is less likely to occur in Europe or the USA in the absence of wild relatives of corn. Further, with wheat and soybean being self-pollinating crops, the risk of transgene, moving to nearby weeds, is minimal. However, some risk is there for GM wheat in the USA as they do have wild relatives of wheat.

14.4.2.3 Contamination of Environment with GM Proteins

Many plants leak chemical compounds into the immediate environment through their root and pollen or from field leftover of plant material after the harvest. This unintentional release of the active proteins into the surrounding is a potential environmental problem with GM crops. It is important to be able to monitor the extent of these losses, because the proteins are found to be toxic to conventional crop-associated microorganisms. Further, through nematodes they could find their way into streams and rivers and may threaten other organisms.

Bt corn roots seep out Bt toxin into the soil. This toxin when bound to soil components is protected from degradation, persists in soil for long time (200 days), and has the ability to kill insect larvae present in soil (Saxena and Stotzky 2001). This seepage of the toxin is only of advantages if soil-living insects are the target. The samples of Quebec's Saint Lawrence River sediments near a field of Bt corn were shown to contain five times more toxin than samples taken from water drainage and sediments around the field indicating the building up of toxin over a period of time on the riverbed. However, conclusive research is necessary to support the results of such results. Also it is not clear how the leakage of toxin in the soil from roots of GM crops might affect nontarget insects and microorganisms inhabiting the soil. The fact is that GMOs have the potential to provide numerous advantages, yet they are still being shown in negative lights by anti-GMO activists who are using arguments without any scientific background.

14.4.2.4 Reductions in Pesticide Spraying

The selling point of the GM crops by the proponents was that the new crop varieties would reduce the use of pesticides as the plant itself produces a toxin that kills major insect pests (Federoff et al. 2010; Carpenter 2001). Thus, if the field is sprayed with the herbicide (Roundup Ready) that kills every other plant in the field except crop transgenic for Roundup Ready, then only small quantity of this herbicide would be needed to keep crop free from weeds. However, opponents of life-saving transgenic technology acknowledge the reduction in pesticide consumption to the following reasons: first, the variation in the population of other insect pests influences the amount of spraying; second, the introduction of new more potent insecticidal active ingredients that are effective at progressively lower rates of application and the use of older insecticides decline; and third, with the development of insect resistance to a chemical, farmer may switch to newer chemicals as they are made available. Thus, because of several factors affecting the amount of pesticide that is sprayed, it is difficult to say that the introduction of Bt corn varieties is solely responsible for the change in pesticide use pattern.

14.5 Biosafety

Cultivation of GM crops is changing the farming practices, chemical application, and land usage. Despite the potential benefits of transgenic crops, public have negative apprehension about the possible impact on environment, agronomy, economy, and ethics. Further, evaluating the short- and long-term impact of these GM crops on the environment is an important hurdle for safe release in areas with rich genetic biodiversity. Biosafety protocols ensure adequate level of protection to minimize the perceived risks to environment and human health in managing live modified organisms developed through modern biotechnology. In the developing years of transgenic technology, a major role was played by molecular biologist. With increase in number of field releases of transgenic plants, biosafety implications attracted global attention, and ecologists and environmentalists too joined to address the perceived ecological risks.

Soon it became very imperative to have expert bodies to be responsible for formulation and implementation of regulatory rules on genetic modification applications for approval of new planting material and genetically engineered foods, nationally and internationally. With growing commitment of people for sustainable agricultural development, the Convention on Biological Diversity (CBD) was established. Biodiversity safety with respect to its sustainable use and conservation is the key elements in the Convention on Biological Diversity (CBD) treaty. CBD attends to the issues of theoretical as well as probable potential exploitation of modern biotechnology, while simultaneously safeguarding against potential risks from its usage. The Cartagena Protocol on Biosafety adopted in 2000 is an international

treaty under the Convention on Biological Diversity, describing the movements of living modified organisms (LMOs) across borders of different countries. Under this treaty, the exporting countries have an obligation to display all the relevant information about the materials or products that are genetically modified, so that the importing countries make appropriate and informed proclamation. Endorsed and signed by 130 countries, the treaty sets out effective methods for risk assessment and management. The Advance Informed Agreement (AIA) procedures look into the methods adopted to keep an account of the intentionally introduced transgenic plants into the environment which may threaten biodiversity, capacity building, and technology transfer. It is imperative for the participating countries to take necessary and appropriate administrative actions and implement defined obligations to eliminate the negative consequences which may cause risks to animal and human health. These countries are also involved in developing common National Biosafety Frameworks (NBFs) for the development and utilization of GM products. NBF is a combination of administrative, technical, and legal policies that are developed to ensure an adequate protection in case of transfer, handling, and use of LMOs which may have adverse effect on the sustainable use and conservation of biodiversity. The Global Environment Facility of the United Nations Environment Programme (UNEP-GEF) has supported these participating nations since 2001 to develop their own NBF. It formulates biosafety guidelines to conduct research on biotechnology and genetic engineering and also take care of the transboundary movement of genetically modified (GM) crops and their products. Synchronization of each and every regulation at the regional, national, as well as international level to building capacities is critical for the coordinated implementation and generation of the benefits of biotechnology to farmers, researchers, and consumers (Singh et al. 2014). Various institutions over the world are actively supporting this cause. IFPRI (International Food Policy Research Institute) compiles the research implications of genetic engineering technologies and policy to alleviate poverty problems in countries under development. The Centre for the Application of Molecular Biology to International Agriculture (CAMBIA) has been commissioned by most of the developing nations to develop a database aiming at the technology ownership. The technology ownership determines the extent of freedom enjoyed by the scientists for manipulation of particular germplasm and crops. An information initiative of United Nations International Development Organization (UNIDO) named as BINAS (Biotechnology Information Network and Advisory Service) serves as a center for disseminating information of biotechnology laws and regulations.

14.5.1 Levels of Biosafety

The goal of biosafety is to prevent dissemination of the modified species (transgenic plants) outside its growing area. Biosafety levels are defined in terms of using specific laboratory practices and techniques, safety equipments, and laboratory

facilities required for different categories of plants and plant-derived products based on their nature to cause damage to environment and living beings. The NIH Guidelines specifically address the effective containment of recombinant DNA. These recommendations, however, are equally relevant to nonrecombinant research. Biosafety level (BSL)1-plants (P), BSL2-P, BSL3-P, and BSL4-P are recommended for research involving transgenic plants in Appendix P of NIH Guidelines.

BSL1-P is the moderate level of containment for biological experiments. It is designed to contain the possible survival, transfer, or dissemination of recombinant DNA into the environment. This containment level is also extrapolated to all such experiments where there is no recognizable and predictable risk to the human health and environment in case of accidental release. It supports the accepted scientific practices for conducting research in an ordinary greenhouse or growth chamber facilities and integrates accepted procedures for effective pest control and cultural practices. BSL1-P facilities and procedures also provide a protected environment, modified for the propagation of plant-associated microorganisms, and at the same time also adequately curtail the potential for release of live plants, plant parts, and microorganisms associated with them.

BSL2-P is designed to ensure a higher level of containment for experiments related to plants and certain associated organisms in which there is an evident possibility of survival, transmission, or dissemination of genetically modified organism containing recombinant DNA. The consequence of such an inadvertent release, however, has a predictably minimal biological impact. BSL2-P depends upon accepted practices for conducting scientific research in greenhouses with organisms infesting or infecting plants. This facility minimizes or prevents unintentional release of plants within or surrounding the greenhouse.

BSL3-P and BSL4- P describe higher containment conditions for research with plants and plant pathogens and other organisms that require special containment because of their known potential for significant damaging effect on managed or natural ecosystems. As BSL2-P, BSL3-P also relies upon accepted scientific practices for conducting research in greenhouses with organisms infesting or infecting plants in a manner that minimizes or prevents unintentional release of plants within or surrounding the greenhouse. BSL4-P describes facilities and practices to provide containment of certain exotic pathogens of plant.

14.5.1.1 Biological Containment Practices (Plants)

Containment is crucial in transgenic plant research in the labs, greenhouses, and growth chambers. The containment principles, practices, and facilities aim to minimize the possibility of an unforeseen injurious effect on organisms and ecosystems outside of the experimental facility. The inadvertent spreads of a hazardous pathogen from a greenhouse facility to nearby agricultural crop and the inadvertent introduction and establishment of an organism in a new ecosystem are serious threat. The effective containment facility ensures prevention of transgenic interbreeding

with native species, decontamination or inactivation of transgenic plant waste prior to disposal, containment of species that could have detrimental impact on local and agriculturally important species, and control of insect vectors and seeds and pollen of transgenic crops from dispersal and pollination of other transgenic crops. All researchers working with transgenic plants must be register with the IBC, determine the appropriate biosafety level for the work to be performed by them, and have standard operating procedures in place for storage, transport, and handling of GM seeds and plant materials with proper labeling and segregation of transgenic and non-transgenic plant materials and curtailing the dissemination of genetic material in the environment.

The level of containment is determined using the knowledge of the organisms and judgment based on accepted scientific practices. Any genetic modification that has the objective of increasing pathogenicity or converting a nonpathogenic organism into a pathogen should be done in high level of containment, depending on the organism, its mode of dissemination, and its target organisms. According to NIH Guidelines, the experiments falling under Section III-E require Institutional Biosafety Committee notice simultaneously with the start of the experiment. Experiment falling under Section III-D requires Institutional Biosafety Committee approval before its initiation. BSL1-P is recommended for all experiments with recombinant DNA-containing plants and plant-associated microorganisms (excluding those covered under Section III-D). For example, plant transformation experiments using recombinant *Agrobacterium* as the genetic modification are not expected to increase undesirable characteristics therefore, BSL1-P can be used.

Experiments requiring BSL2-P or a higher level of containment involve plants that are harmful weeds or that can interbreed with harmful weeds in the immediate geographic area or have recognized potential for rapid and widespread dissemination or for severe harmful impact on managed or natural ecosystems.BSL2-P or BSL1-P+ biological containment is recommended for:

1. Plants modified by recombinant DNA that are noxious weeds or can interbreed with noxious weeds in the immediate geographic area [Section III-E-2-b-(1)].
2. Plants in which the introduced DNA represents the complete genome of a nonexotic infectious agent [Section III-E-2-b-(2)].
3. Plants associated with recombinant DNA-modified nonexotic microorganisms that have a recognized potential for severe harmful effect on managed or natural ecosystems [Section III-E-2-b-(3)].
4. Plants associated with recombinant DNA-modified exotic microorganisms that have no recognized potential for serious harmful effect on managed or natural ecosystems [Section III-E-2-b-(4)].
5. Experiments with recombinant DNA-modified arthropods or small animals associated with the plants. These also include recombinant DNA-modified microorganisms associated with arthropods or small animals, and the recombinant DNA-modified organisms have no recognized potential for harmful effect on managed or natural ecosystems [Section III-E-2-b-(5)].

BSL3-P or BSL2-P+biological containments are recommended for:

1. Experiments involving most exotic infectious agents with recognized potential for serious harmful effect on managed or natural ecosystems when recombinant DNA techniques are associated with whole plants [Section III-D-5-a]
2. Experiments involving plants containing cloned genomes of readily contagious exotic infectious agents with recognized potential for serious harmful effect on managed or natural ecosystems in which a possibility of reconstituting the complete and functional genome of the infectious agent by genomic complementation in plants exists [Section III-D-5-b]
3. Experiments with microbial pathogens of insects or small animals associated with plants, if the recombinant/synthetic nucleic acid molecule-modified organism has a recognized potential for serious harmful effect on managed or natural ecosystems [Section III-D-5-e]

BSL3-P is recommended for experiments relating to potent vertebrate toxins encoding sequences to be established into plants or plant-associated organisms [Section III-D-5-d].

14.5.1.2 Biological Containment in the Greenhouse

Greenhouses are the containment structures created by using transparent or translucent covering and provide a controlled environment for growing plants or plant-associated organisms. The plant-associated organisms include fungi, viruses, bacteria, protozoa, nematodes, insects, mites, and others. The physical and biological containment conditions and practices in greenhouse are carried out as specified in Appendix P of the NIH Guidelines. Appendix P applies when the research plant number, size, or growth requirements prevent the use of laboratory containment conditions (in growth chambers, tissue culture rooms, or open benches) as described in Appendix G, Physical Containment. It enlists the physical and biological containment procedures and management protocols applicable to each of four biosafety levels, designated as BSL1-P, the lowest level of containment through BSL4-P, the highest level.

14.5.1.3 Biological Containment of Plants

Growing plants need to be contained to prevent the effective spreading of genetic material. This can be achieved by covering or removing the reproductive structures to prevent pollen dissemination at flowering and seed dispersal at maturity, harvesting plant material prior to sexual maturity, and removing reproductive structures by using male sterile strains. Further it should be ensured that the cross-fertile plants are not growing within the known pollen dispersal range of the experimental plant or induce flowering in experimental plants at a time of year when no flowering occurs in cross-fertile plants, within the normal pollen dispersal range.

Various commercial containment facilities are available, or inexpensive systems can be constructed with easily available disposable plastic sheeting. These systems contain seeds, soil, and plant parts resulting in less housekeeping, less contamination between shelves, and better humidity control resulting in less watering of plants.

14.5.1.4 Biological Containment of Microorganisms Associated with Plants

Prevention of dissemination of plant-associated microorganisms beyond the confines of the greenhouse is very important. This can be achieved by confining all operations, from manipulation of genetic material to injection thereof in microorganisms that limit replication or reproduction of viruses and microorganisms, and confine these injections to internal plant parts or adherent plant surfaces. Further ensuring the nonexistence of the organisms that can serve as hosts or help in the transmission of the virus or microorganism within the longest distance that the airborne virus or microorganism may be expected to cover to be effectively disseminated by these hosts or vectors may curtail the contamination. Containment can naturally be achieved if experiments are conducted at a time of year, when plants that can serve as hosts to plant-associated microorganisms are either not growing or are not susceptible to active infection. The use of microorganisms that are genetically modified to minimize their survival outside of the research facility and whose natural mode of transmission requires damage of the target organism, or assures that unintentional release is unlikely to initiate productive dissemination of organisms outside of the experimental facility, is also effective containing methodology for plant-associated microorganisms.

14.5.1.5 Biological Containment of Macroorganisms

The spreading of arthropods and other related small animals can be prevented by using flight-impaired, nonflying, or sterile arthropods and/or by using sterile or nonmotile strains of small animals. Dissemination can be prevented by conducting experiments at a time of year that are hostile for escaping organisms, by using animals that have an obligate involvement with a plant that is absent from the dispersion range of the organism, or by preventing the escape of organisms present by chemical treatment in runoff water or evaporation of runoff water.

14.5.2 Biosafety Boards Operating in India

The Indian biosafety regulatory system has evolved in response to scientific advances and the growing concerns of public, scientists, and government organizations about the biotechnology products. The Indian regulatory system ensures that

GM crops pose no major risk to food quality and safety, environmental safety, and agricultural production with no adverse economic impacts on farmers. The GMOs and products thereof are regulated articles in India in view of potential risks to human health and environment. The first set of rules is formulated under the Environment Protection Act (EPA), 1986, by the Ministry of Environment and Forests (MoEF) to stop the indiscriminate use of GMOs and referred to as Rules 1989. It provides "Rules for manufacture, use, import, export and storage of Hazardous microorganisms/Genetically engineered organisms or cell." The act ensures protection and improvement of "environment," which includes "water, air and land and the inter-relationship, which exists among and between water, air and land, and human beings, other living creatures, plants, microorganism and property." The Rules 1989 cover (1) manufacturing, import, and storage of microorganisms and gene technological products; (2) genetically engineered organisms/microorganisms and cells and correspondingly to any substance, products, food material, etc., of which such cells, organisms, or tissues form part; and (3) newer transgenic technologies along with hybridization of cell and genetic engineering.

The Ministry of Environment and Forests (MoEF) and the Department of Biotechnology (DBT) are the two apex bodies of the Government of India responsible for regulation of rDNA products and implementation of the rules regarding GMOs. MoEF has developed guidelines for the manufacture, import, use, research, and release of GMOs as well as products produced from GMOs to ensure the safety of human beings and the environment. Safety guidelines were developed by DBT in 1990 for carrying out research in the field of biotechnology, field trials, and commercial applications. Separate guidelines are developed by DBT for research involving transgenic plants in 1998 and for clinical products thereof in 1999. Activities involving GMOs are also covered under other policies such as the Drugs and Cosmetics Act (8th Amendment), 1988; the Drug Policy, 2002; and the National Seed Policy, 2002. India has ratified the international regulatory framework Cartagena Protocol which is a sequel to the convention on biodiversity enacted in 1992 at the Rio Summit, in 2003.

The Rules 1989 also define the competent authorities and the composition of such authorities for managing various aspects of the rules. There are six competent statutory bodies under the DBT and state government for the implementation of regulations and guidelines across the country as listed below:

1. Institutional Biosafety Committee (IBSC): It is established under the institution engaged in GMO research to oversee such research and to streamline them with the RCGM for the regulation.
2. District Level Committee (DLC): These committees have a major role in monitoring the safety regulations in installations engaged in the use of GMOs/dangerous microorganisms and their applications in the environment.
3. State Biotechnology Coordination Committee (SBCC): These committees have a major role in monitoring. SBCC also has powers to inspect, investigate, and take penalizing action in case or violations of statutory provisions.

4. Genetic Engineering Approval Committee (GEAC): It was established under MoEF and is the apex body. GEAC was structured under Rules 1989 for approval of activities involving large-scale use of unsafe microorganisms and recombinant organisms in research and commercial production with respect to environmental safety. Any release of GMO and its products thereof into the environment which include the experimental field trials (Biosafety Research Level trial-I and II, known as BRL-I and BRL-II) should have approval by GEAC.
5. Review Committee on Genetic Manipulation (RCGM): The RCGM is established under the Department of Biotechnology (DBT), Ministry of Science and Technology, to monitor the safety-related aspects in ongoing scientific research activities and projects (including small-scale experimental field trials) and specify the protocols to be followed to implement regulatory procedures with respect to activities involving GMO research, usage, and applications in research and industry, to ensure environmental safety bring out manuals and guidelines.
6. The Recombinant DNA Advisory Committee (RDAC): RDAC acts as an advisory body which reviews the biotechnology development at international as well as national levels. The recommendation on suitable and appropriate safety regulations in recombinant DNA research, usage, and applications, from time to time, is used to frame Indian policies for environmental safety.

Thus various agencies are involved in approval of new transgenic crops. RCGM monitors ongoing research activities in GMOs and small-scale field trials at national level. GEAC authorizes large-scale field trials and environmental release of GMOs. The Recombinant DNA Advisory Committee (RDAC) monitors the developments in biotechnology at international as well as national levels and proposes appropriate recommendations. The State Biotechnology Coordination Committees (SBCCs) coordinate the research activities involving GMOs in the state with the central ministry. SBCC inspects, investigates, and takes punitive action if violations occur. Similarly, District Level Committees (DLCs) monitor the safety regulations in installations engaged in the use of GMOs in research and application, at district level.

14.6 IPR in Plant Biotechnology

Intellectual property rights (IPR) are about protection of rights of person creating a new and original concept in the global context. These exclusive rights are awarded by the government and define the possession in similar ways as for all tangible things such as house, land, vehicle, and so on. This restrains others from using the property without consent or permission of the creator. Further these rights are awarded under certain laws and valid over a fixed time period. After the end of validity period of this protection, others are free to use the intellectual property (IP).

Intellectual property is thus described as the property which originates through the creative effort of the inventor, produced or originated by human skill, intelligence, labor, and efforts, and gives the owner a right to such property.

IP rights is a body of law that is developed to give creative people, who have disclosed their work for the benefit of mankind, an ownership right over their creation. These rights protect the work of innovators from being copied or imitated without their consent. Intellectual property plays an important role in growth of economic interests of a country. The technology developed, research made, or invention done in a country is protected which in turn strengthens the economy of that country.

Except geographical indications and trademark, IPR are for the fixed time period. Geographical indications and trademark have indefinite life even after the stipulated time by paying official fees. IPR have to be renewed after the expiry period to keep them enforced except in case of copyright and trade secrets. Except copyright, IPR are largely territorial rights. Copyright is a global right. IPR can be held only by legal entities, self-governing entities having the right to purchase and sell property. These rights can be used, assigned, sold, licensed, and gifted. IPR can be enjoyed in more than one country, simultaneously.

14.6.1 IPR Categories

Presently the following categories of IPRs are recognized the world over:

(a) Patents
(b) Copyrights
(c) Trademarks
(d) Designs for industrial use
(e) Lay out design of integrated circuits
(f) Geographical indications
(g) Protection of undisclosed informations
(h) Protection of new plant variety and farmers' rights

Significance of IPR

- Launching new products, processes, and services
- Helping to take lead in the market
- Licensing and assignments
- Joint ventures and mergers
- Takeovers
- Enhancing market value
- Raising funds
- Strategic purpose

14.6.2 IPR in Plant Biotechnology

Research and development in the plant biotechnology and agricultural sector is unique among industries, and in relation to other industries, research and innovation in plant biotechnology and agriculture are far more geographically dispersed. Research and development in plant biotechnology is dominated by public research institutions, and nearly two thirds of share in research and development in these areas are contributed by these institutions. Private sector involvement in innovation in these areas is a recent phenomenon. Presently public as well as private sectors are involved in generation of IPRs in these areas.

Grant of intellectual property protection to living organisms and plant varieties is contentious and faces stiff opposition from environmentalist and other groups opposed to genetic modification/engineering ostensibly on account of biosafety and public health issues as well as on the core issue of grant of IP protection on living organisms being unsuitable and against the laws of nature. There is considerable concern that the intellectual rights protection might adversely affect food sovereignty and security as well as result in abuse of competition by creation and perpetuation of a small number of ever-growing very large players controlling technology and production rights over agricultural produce.

Presently developments in agriculture and plant biotechnology are protected by following IPR categories:

14.6.2.1 Patents

Patents are the legal rights that protect processes and product inventions which are made from tangible things. They give exclusive right to owner to decide its usage and discourage others to use invention as claimed in the document, describing the patent. Innovations in agricultural biotechnology such as transformation processes, transformation vehicles and other vectors, and components of vectors (origin of replication, promoters, genes of interest, and markers) are covered under utility patents.

Patentability of plants and plant varieties varies in different countries. Laws in the USA are liberal in this regard. US patent laws allow patentability of transgenic plants and their parts under "plant patent." The US government grants a plant patent to an inventor (or his assigns or heirs) who has invented or discovered and reproduced a distinctly new plant variety asexually, excluding tuber-propagated plant or a plant found in wild state. The grant is for 20 years from the application filing date and protects the right holder to discourage others from using or selling the new plant or asexually reproducing it. As the plant patents are exclusive patent in agriculture, they must also satisfy the general patentability requirements. Thus a plant invented or discovered by applicant and has been found to be stable after asexual reproduction should be the subject matter of the patent application. Indian and European patent statutes, however, do not allow protection of plants under patents.

14.6.2.2 Plant Breeders' Rights (PBR)

Plant breeders' rights are the protection provided to breeders by member nations of the International Union for the "Protection of New Varieties of Plants" (*Union Internationale pour la Protection des Obtentions Végétales*, UPOV) founded in 1961. After adopting the convention in 1961 in Paris, it was revised in 1972, 1978, and 1991 with an objective of encouraging the new plant variety development programs for the benefit of public in general.

Under the rules established by UPOV, to grant plant breeders' rights, the new variety must be:

(a) Novel, that is, it must not have been previously marketed where the country rights are applied for.
(b) Different from other available varieties.
(c) Exhibiting homogeneity.
(d) Stable with respect to unique traits and the plant remains true even after repeated cycles of propagation.

Any of the methods, conventional breeding techniques or genetic engineering, could be used to develop new plant variety (legally defined) for which protection is filed.

14.6.2.3 Indian Legislation on Protection of Product and Services in Agri-Biotech Sector

The regulatory and policy framework in India with respect to protection of the new plant varieties did not exist in the past because of sole control and dominance of the public sector. The private sector played minimal role. At the advent of green revolution, the need was felt to give farmers more incentives and encouragement, to use seeds of high-yielding crop varieties.

Now there are many legislations and statues which are enacted by the Indian legislature involving Ministry of Agriculture/ICAR and Ministry of Environment and Forests. The **Protection of Plant Variety and Farmers Right Act, 2001** (PPVFR Act), under the Ministry of Agriculture/ICAR, is an act of the Parliament of India formed to provide for the effective system for protection of plant varieties, to protect the rights of farmers and plant breeders, and to encourage investments in development and cultivation of newer plant varieties.

Essential requirements to be eligible for registration for new plant variety under the Protection of Plant Variety and Farmers Right Act, 2001, is that it must conform to the criteria of novelty, distinctiveness, uniformity, and stability (NDUS), as described in Section 15 (1)–(3) of the act.

14.7 Summary and Future Prospects

Extremely strong discussion about the potential benefits and danger associated with GM crops is going on in various parts of the developing world. Despite the current uncertainty over GM crops, this technology, with its great potential to create economically important crop varieties, is drawing good amount of attention. The two broad groups are formed due to this a pro-GM group comprising of agro-biotechnology institutions, many government departments, and seed breeding and marketing industries and an anti-GM group comprising of consumer and environmental organizations. The farmers' associations and the media are split between the two sides. The principal issues of disagreement are the extent of yield enhancement, pesticide and herbicide usage decrease, impact on the ecology and biodiversity, animal and human health, the socioeconomic position and livelihood of small farmers, and finally the ownership and control of genetic resources and trade. Thus, the benefits of advancement in agricultural biotechnology can be effectively harnessed by appropriate knowledge of the areas like biosafety, production patterns, biodiversity, and intellectual property rights.

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